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Activation of Nucleotide Binding Oligomerization Domain Containing Protein 1 in 3T3-L1 Adipocytes: Effects on Adipocyte Differentiation and Lipolysis

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To the Graduate Council:

I am submitting herewith a thesis written by Jaanki Shamb Purohit entitled "Activation of Nucleotide Binding Oligomerization Domain Containing Protein 1 in 3T3-L1 Adipocytes: Effects on Adipocyte Differentiation and Lipolysis." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutritional Sciences.

Ling Zhao, Major Professor

We have read this thesis and recommend its acceptance:

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Activation of Nucleotide Binding Oligomerization Domain Containing Protein 1 in 3T3-L1 Adipocytes: Effects on Adipocyte Differentiation and Lipolysis

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DEDICATION

I dedicate this work to my loved ones Shamb, Trupti, and Rucha Purohit and to Sean Hoer. They have encouraged and supported me to achieve my goals from hundreds of miles away.

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ABSTRACT

Obesity, defined as having excess adipose tissue, is associated with chronic inflammation. Adipose tissue is made up of many cell types, including preadipocytes and adipocytes. Both preadipocytes and adipocytes express pattern recognition receptors that play important roles in innate immunity. Two families of pattern recognition receptors that have been studied in adipose tissue are Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Activation of TLR2 and TLR4 has been shown to lead to proinflammatory response in adipocytes, which is shown to suppress adipocyte differentiation and stimulate lipolysis, one of the major physiological functions of adipocytes. However, the effects of NOD activation on adipocytes have not been studied. Here, we show that activation of NOD1, but not NOD2, by synthetic ligand, suppresses 3T3-L1 adipocyte differentiation shown through Oil-Red-O stained morphology, lipid accumulation, and attenuated gene expression of transcriptional factors PPAR gamma and C/EBP alpha and adipogenic markers (adiponectin, leptin, SCD, FABP4). Moreover, we show that activation of NOD1 by synthetic ligand C12-iEDAP stimulates lipolysis in 3T3-L1 adipocytes in a time and dose-dependent manner. The effects of C12-iEDAP are attenuated by knockdown of NOD1, demonstrating specificity. Additionally, inhibition of NF-kappa B and protein kinase A/hormone sensitive lipase via pharmacological inhibitors attenuate the lipolytic effects of C12-iEDAP. NOD1 activation also suppresses lipid droplet coating protein perilipin expression. Overall, our results suggest that NOD1 represents a novel target for adipose inflammation for obesity treatment and prevention.

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INTRODUCTION

The dynamic role of adipose tissue

The defined physiological role of white adipose tissue is expanding along with the prevalence of obesity. Obesity is defined as having an excess of body fat. Comorbidities associated with obesity are on the rise, including diabetes, cardiovascular disease, kidney disease, and cancer--each the resulting from various sources of inflammation (1).

White adipose tissue is made up of preadipocytes, adipocytes, lymph nodes, stromal-vascular tissue, and tissue matrix. Adipose tissue has several physiological roles, the first of which is energy storage (2). As energy availability exceeds energy expenditure, insulin acts on adipose tissue to store triglycerides in the lipid droplet of the adipocyte. Additionally, preadipocytes begin to differentiate into adipocytes to accommodate excess energy. Eventually, new preadipocytes differentiate and are filled with triglycerides, the cycle continues and leads to overweight and obesity(3).

The second major function of adipocytes is to release energy when energy expenditure exceeds availability. The triglycerides located inside the adipocyte are broken down into free fatty acids and glycerol to free energy stores for tissues throughout the body—this process is known as lipolysis. The classical lipolysis pathway outlines that lipolysis is triggered by catecholamine stimulation. Adrenaline and noradrenaline stimulate β-adrenergic receptors to eventually stimulate hormone sensitive lipase (HSL) and results in lipolysis (4). However, studies have found that

obese subjects tend to have elevated circulating free fatty acid levels, despite excess energy intake (1, 5).

Adipose tissue has also been shown to have endocrine function through the secretion of proteins such as adiponectin and leptin into circulation. Adiponectin, a functional protein secreted by adipocytes, skeletal and cardiac muscle cells, and circulates at levels as high as 10mg/mL in the bloodstream (6). Those with higher levels of circulating adiponectin have less visceral obesity and insulin resistance (7). Adiponectin has also been shown to block NF-κB transcription in macrophages in endothelial cells, providing anti-inflammatory function (8). In terms of insulin resistance, adiponectin has been shown to decrease hyperglycemia, reduce circulating free fatty acids, and increase insulin sensitivity when administered in obese animals (6). Leptin, also secreted by adipocytes, is a key regulator of energy homeostasis and is a mediator in the immune response (9). Leptin is well known for playing a role in appetite control, and in non-obese subjects, circulating leptin levels correlate with adiposity (6).

Previously presented as an energy storage unit, white adipose tissue has an established role in endocrine function, and perhaps more surprisingly, innate immunity (10). Innate immune receptors such as Toll-like receptors and nucleotide oligomerization domain-like receptors (NOD-like receptors) are expressed in adipocytes, and trigger pathways such as nuclear factor-kappaB (NF-κB) and mitogen activated protein kinase (MAPK) to cause cytokine and chemokine secretion. Adipocytes secrete several proinflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), macrophage inflammatory protein-2 (MIP-2), and

monocyte chemoattractant protein-1 (MCP-1) (11, 12). Cytokine and chemokine secretion lead to disrupted physiological pathways including adipocyte differentiation and lipolysis. Disrupted adipocyte differentiation and lipolysis lead to elevated circulating free fatty acids which interfere with insulin signaling and act as endogenous ligands for inflammatory receptors. Continuously elevated circulating free fatty acids are a major contributing factor to insulin resistance, cardiovascular disease, and systemic inflammation (11, 13, 14).

Adipocyte proliferation and differentiation

The development of adipocytes begins in the vascular stroma of adipose tissue or bone marrow with a pluripotent stem cell that can transform into myocytes, osteocytes, chrondrocytes, or adipocytes (15). Adipogenesis (Figure 1), the process by which mesenchymal stem cells proliferate and commit to the adipocyte line, is promoted by activation of bone morphogenetic proteins BMP4 and BMP2 (16, 17) and Wnt (17, 18). Wnt signaling promotes commitment into the adipocyte line but inhibits adipocyte differentiation (19) while hedgehog (Hh) (20) inhibits commitment to adipocyte lineage. A variety of signaling factors determine whether the pluripotent cell becomes an adipocyte, myocyte, osteocyte, or chrondrocyte and each signal can promote one lineage while inhibiting another. Once the mesenchymal stem cell is committed to the adipocyte lineage, a preadipocyte is created. The term proliferation in relation to cell-line studies generally signifies the replication and subsequent differentiation of preadipocytes (2).

Differentiation of adipocytes has been studied extensively through the use of established cell lines. Preadipocyte cell lines such as 3T3-L1 have been shown to mimic differentiation of mouse embryonic fibroblasts in cell culture (21) and become viable adipocytes that are functional when implanted into immune deficient mice (22). Induction of differentiation of 3T3-L1 preadipocytes occurs by first reaching G_1 phase of the cell cycle, then inducing differentiation using a differentiation cocktail often consisting of a high dose of insulin, cAMP inducer, and dexamethasone. Within 16-20 hours after adding the cocktail, the morphology of the preadipocytes go through mitotic clonal expansion and take a fibroblastic shape. The cells then exit the cell cycle and accumulate triacylglycerol (TAG) and become round, lipid-filled adipocytes (23). As the adipocyte accumulates TAG, many functional proteins are expressed, such as insulin receptors, leptin, and insulin-responsive glucose transporter GLUT4 (24). Key transcription factors include CCAAT/enhancer-binding protein-α, β and –δ (C/EBP) and peroxisome proliferator-activated receptor gamma (PPARγ). C/EBPβ activates the expression of PPARγ and C/EBPα, which then coordinate the activation of numerous genes resulting in the adipocyte phenotype (25). These transcription factors work in harmony to regulate adipocyte marker genes such as stearoyl COA desaturase (SCD), fatty acid binding protein 4 (FABP4) which play a role in the incorporation of triglycerides in the lipid droplet of adipocytes. Other adipocyte marker genes include lipoprotein lipase (LPL), which is a regulator of adipocyte differentiation and perilipin (PLIN), which regulates HSL-mediated lipolysis (26, 27).

In summary, in times of excess energy, the body sets off a series of transcription factors to promote growth and differentiation of the preadipocytes into a fully mature adipocyte with a lipid-filled core (28).

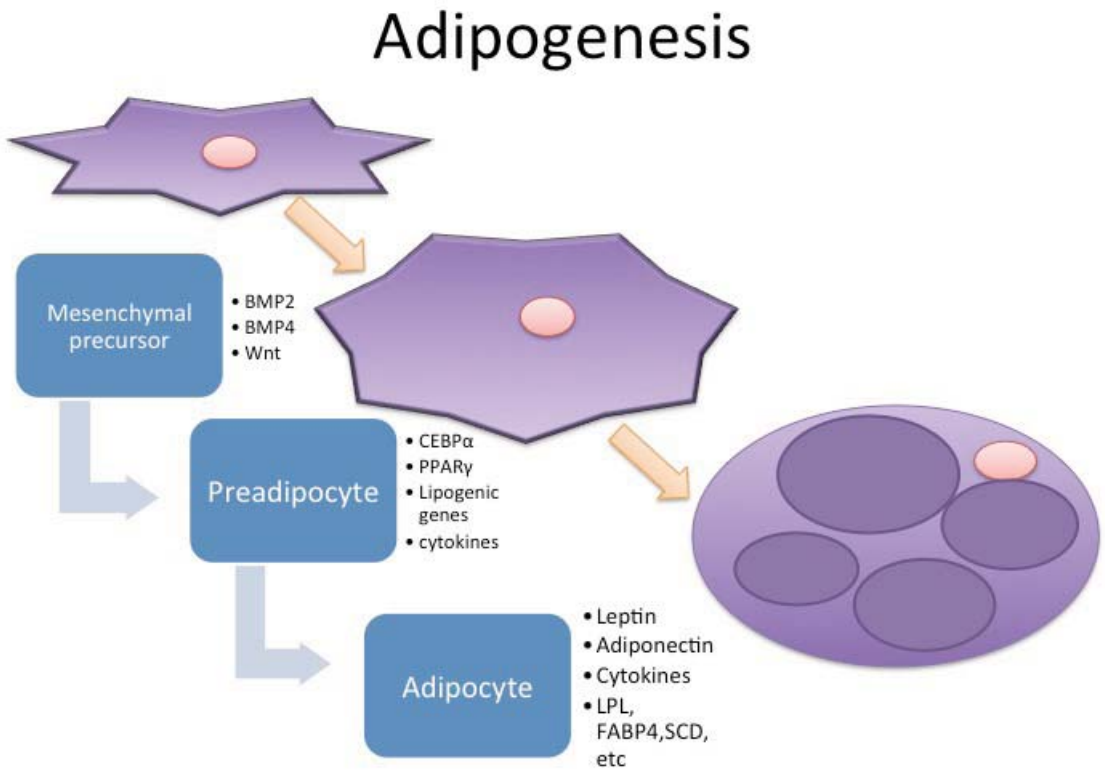

Figure A: Adipogenesis: Adipogenesis begins when the proper signals via the mesenchymal precursor is activated to lead to the development of preadipocytes. Transcription factor activation drives the differentiation process. Lipogenic genes are expressed and cytokines are released to form a lipid-filled adipocyte. Mature adipocytes express and secrete adiponectin, leptin, cytokines and chemokines such as leptin and adiponectin.

Innate immune function of adipocytes

Innate immunity is the first line of defense of the host. Unlike adaptive immunity, the innate immune system does not present long-lasting defense to the host organism. But rather, it provides quick recruitment of defense mechanisms through the secretion of cytokines and chemokines to the invading pathogens or damages which present pattern associated molecular patterns (PAMP or DAMP) (29). Pattern recognition receptors (PRRs) are responsible for recognizing PAMPs and DAMPs—many are expressed in preadipocytes and mature adipocytes. PRRs, such as transmembrane Toll-like receptors (TLRs) and cytosolic Nod-like receptors (NLRs), have been extensively studied (12, 30-37). For example, TLR4 recognizes lipopolysaccharide (LPS) (34). TLR activation leads to activation of NF-κB and MAPK pathways. Activation of these pathways results in proinflammatory gene expression (34, 37, 38). Physiological insults such as an inflammatory environment can significantly alter the expression of integral proteins and transcription factors in the adipocyte, potentially leading to dysfunction.

NOD-like Receptors

The cytosolic nucleotide oligomerization domain receptor (NLR) has a central nucleotide binding domain, an N-terminal interaction domain, and a C-terminal leucine-rich repeat domain (39). Two key members of the NLR family are NOD1 and NOD2 which recognize bacterial peptidoglycan dipeptide, γ-D-Glu-meso-diaminopimelic acid (iE-DAP) (40) or a tripeptide, L-Ala-γ-D-Glu-meso-diaminopimelic acid (41) which is

found primarily in gram-negative bacteria. NOD2, on the other hand, recognizes muramyl dipeptide, MurNAc-L-Ala-D-isoGln (MDP), from both gram-positive and gram-negative bacteria (42, 43). When they are activated by their respective ligands, both NOD1 and NOD2 interact with the Rip2/RICK/CARDIACK kinase through their caspase recruitment domain (CARD) to activate NF-κB (43, 44). Activation of NOD1, like TLR4, also activates the MAPK pathway, resulting in proinflammatory gene expression (45). Activation of NOD1 also suppresses insulin signaling and subsequent glucose uptake in 3T3-L1 adipocytes. MCP-1, TNF-α, and IL-6 are among the released cytokines that are implicated in insulin resistance (46).

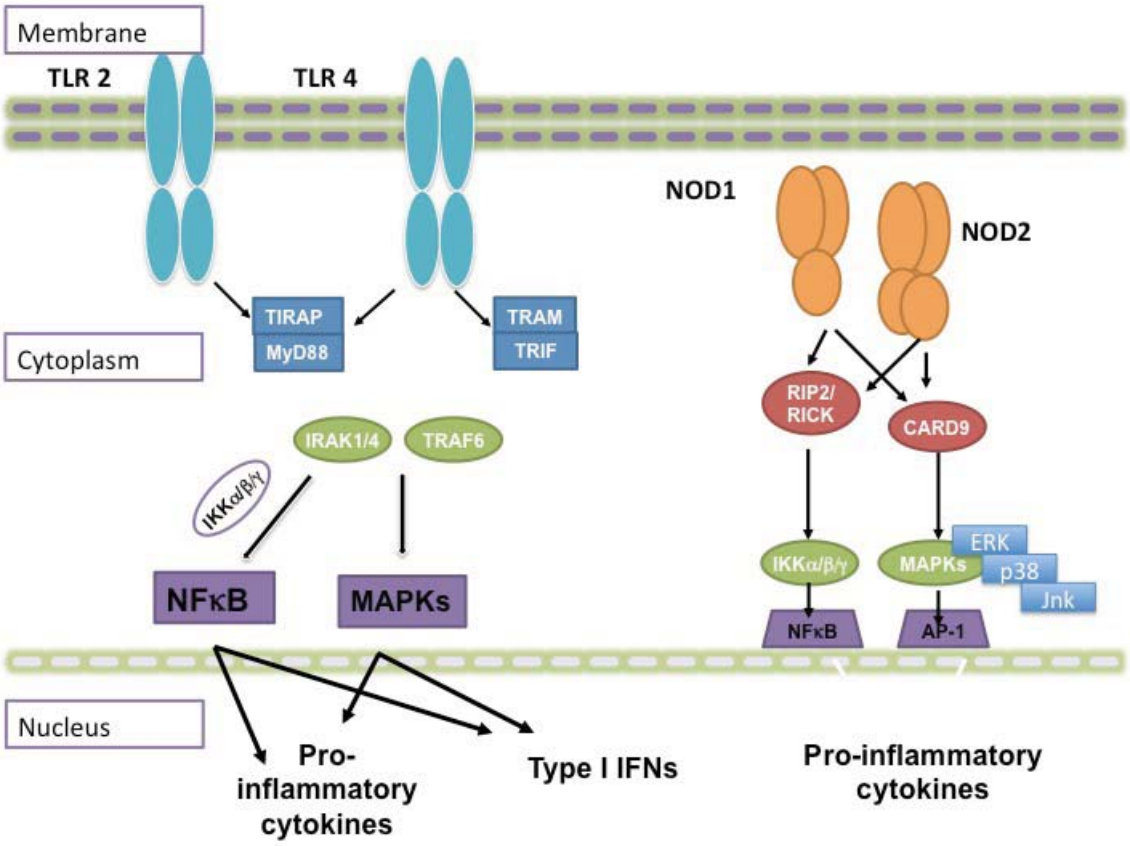

Figure B: Pattern Recognition Receptor Signaling: Recognition of ligand at the cell membrane triggers a signaling cascade resulting in the activation of NF-κB and MAPK pathways. TLR2/TLR4 signaling occurs through the MyD88, which is an adapter protein to activate NF-κB via IRAK signaling. NOD1/2 signaling occurs through the RICK/CARD pathway, activating IKK and MAPK and induces proinflammatory cytokine transcription and release.

Consequences of Inflammation in adipocytes

This research project explores the effect of NOD1-induced inflammation on two major physiological functions of adipose tissue: differentiation and lipolysis. Differentiation, described above, is the process by which a preadipocyte transforms into mature adipocyte to become an energy storage unit and a key player in endocrine function. Another major physiological role of white adipose tissue is the mobilization of energy stores by the process known as lipolysis. Inflammation stemming from adipose tissue is thought to be a major contributing factor to insulin resistance and other complications associated with obesity. Inflammation can interfere with insulin signaling, disrupt lipid storage and breakdown, and suppress adipocyte differentiation. The mechanism through which inflammation occurs and how it affects other metabolic processes remains unclear. However, there is some evidence that TLR activation affects adipocyte differentiation and induces lipolysis. There are multiple sources of inflammation including that from other PRRs, such as NOD1, which contribute to the inflammatory environment. Thus, the focus of this project is the effect of NOD1-mediated inflammation.

Excess energy is stored in white adipose tissue in the form of TAG within the lipid droplet of the adipocyte, a process facilitated by insulin. When energy availability is low, a series of hormones break down the TAG store into free fatty acids (FFA) and glycerol to be used as energy substrates by other tissues. FFAs are not only an energy source, they also function as signaling molecules (47). Chronically elevated FFA alters glucose

and lipid metabolism in skeletal muscle and liver and may lead to insulin resistance (48).

The activation of lipolysis is a multi-step process in which a series of enzymes cleave fatty acids from the glycerol backbone until only FFA and glycerol remain. Lipolysis activation can also occur due to a proinflammatory environment. One cytokine that is expressed in obesity is TNFα. TNFα induces lipolysis by blocking transcription factor PPARγ signals causing adipocytes to de-differentiate (49, 50). Interleukin-6 (IL-6), another proinflammatory cytokine that is elevated in the obese state, can stimulate lipolysis through the extracellular signal-regulated kinase pathway (51).

The resulting FFA can act as signaling molecules and lead to insulin resistance. FFA can also activate macrophages, leading to increased proinflammatory gene secretion in the adipose tissue via MAPK and NF-κB pathways (38).

Although these various lipolytic pathways have been explored in many cell types, it is still unclear if and how inflammation-triggered lipolysis can lead to a cycle of elevated circulating FFA and glycerol. Circulating FFA and glycerol when energy availability is adequate then leads to systemic inflammation and continued lipolysis in a fed state. However, given the fact that obesity and metabolic syndrome are associated with both elevated FFA and glycerol and systemic inflammation, the mechanism must be explored extensively to pinpoint the root cause.

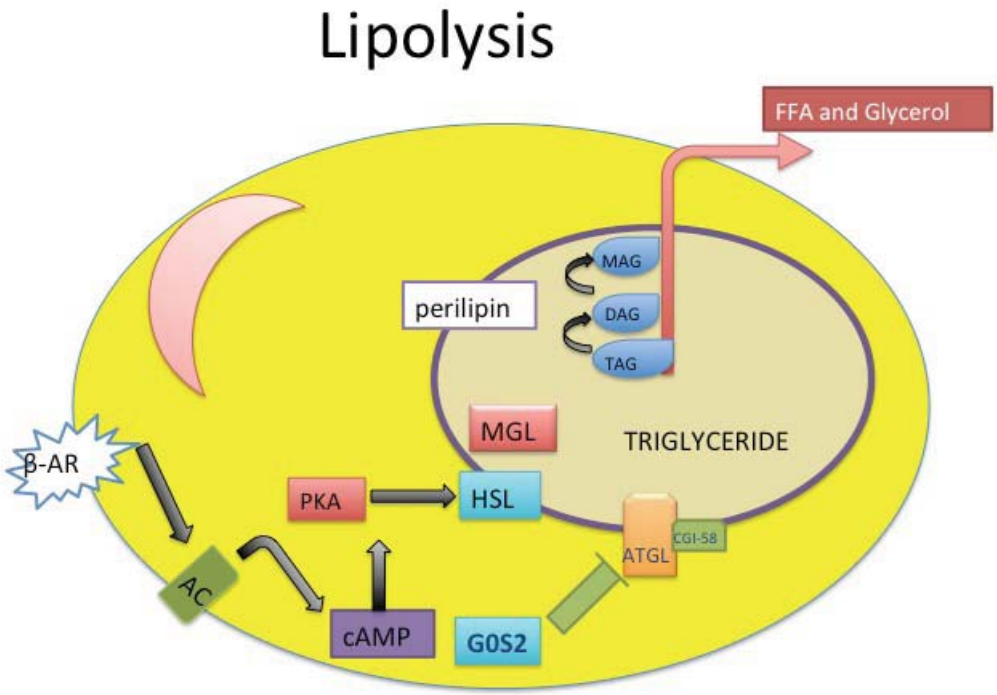

Figure C: Mechanism of Lipolysis: The classical lipolytic pathway begins with catecholamine stimulation of the β-adrenergic receptor, which activates adenenyl cyclase (AC), cyclic AMP (CAMP), and leads to phosokinase A (PKA) activation of the lipid cleaving enzyme, hormone sensitive lipase (HSL). HSL translocates to the lipid droplet to begin the systematic cleavage of the triglyceride.

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CHAPTER I
THE EFFECTS OF NOD ACTIVATION ON ADIPOCYTE DIFFERENTIATION

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ABSTRACT

Obesity is associated with chronic inflammation. Toll-like receptors (TLR) and NOD-like receptors (NLR) are two families of pattern recognition receptors that play important roles in immune response and inflammation in adipocytes. It has been reported that TLR4 and TLR2 activation induce proinflammatory changes that impair adipocyte differentiation. However, the effects of activation of NOD1 and NOD2, the two prominent members of NLR, on adipocyte differentiation have not been studied. Here, we report that activation of NOD1, but not NOD2, by a synthetic ligand dose-dependently suppressed 3T3-L1 adipocyte differentiation as revealed by Oil Red O stained cell morphology, lipid accumulation, and attenuated gene expression of adipocyte markers (PPARγ, C/EBPα, SCD, FABP4, adiponectin). Activation of NOD1, but not NOD2, induced NF-κB activation, which correlated with their abilities to suppress ligand-induced PPARγ transaction. Moreover, the suppressive effect by NOD1 activation was reversed by IκB super-repressor which blocks NF-κB activation. The suppression by NOD1 ligand C12-iEDAP on adipocyte differentiation was reversed by small RNA interference targeting NOD1, demonstrating the specificity of NOD1 activation. In contrast, activation of NOD1 and NOD2 both significantly suppressed adipocyte differentiation of human adipose-derived adult stem cells, demonstrating the species specific effects of NOD activation. In contrast to enhanced leptin mRNA by LPS and TNFα, NOD1 activation suppressed leptin mRNA in adipocytes, suggesting the differential effects of NOD1 activation in adipocytes. Overall, our results suggest that NOD1 represents a novel target for adipose inflammation in obesity.

ABBREVIATIONS

C12-iEDAP, Lauroyl-γ-D-glutamyl-meso-diaminopimelic acid; C/EBPα, CCAAT-enhancer-binding protein alpha; FABP4, fatty-acid binding protein-4; LPS, lipopolysaccharide; MDP, muramyl dipeptide; NOD1, nucleotide-binding oligomerization domain-1; NOD2, nucleotide-binding oligomerization domain-2; PAMP, pathogen-associated molecular pattern; PPARg, peroxisome proliferator-activated receptor gamma; PRR, pattern recognition receptor; SCD, stearoyl-CoA desaturase; TLR2, toll-like receptor 2; TLR4, toll-like Receptor 4; TNF-a; tumor necrosis factor alpha

INTRODUCTION

Obesity is a global epidemic that affects both adults and children. Obesity is associated with increased risk of developing chronic diseases such as insulin resistance, diabetes, cardiovascular disease and certain cancers. Obesity results from not only increased adipocyte cell size but also increased adipocyte cell numbers. New adipocytes are generated by a process known as differentiation. During this process, fibroblast-like preadipocytes are differentiated into mature, spherical, and lipid filled adipocytes. There are more than one hundred molecules up-regulated in the process, including two families of transcription factors, peroxisome proliferator-activated receptor (PPAR) γ and CCAAT/enhancer binding protein (C/EBP) α, which control the induction of the differentiation program, genes involved in lipogenesis (e.g., stearoyl CoA desaturase (SCD), adipocyte specific fatty acid binding protein 4 (FABP4) and adipokines (e.g., adiponectin and leptin) (1, 2).

Accumulating evidence has shown that obesity is associated with chronic inflammation (3, 4). Toll-like receptors (TLR) and nucleotide-oligomerization domain containing protein-like receptor (NLR) are two families of pattern recognition receptors (PRR) that play critical roles in innate immune response and inflammation (5). TLRs are transmembrane receptors composed of extracellular leucine-rich repeat (LRR) motifs, and a cytoplasmic toll/interleukin-1 receptor (TIR) homology domain. So far, 10 and 12

functional TLRs have been identified in humans and mice, respectively (6). NLRs are a family of cytosolic sensors that play important roles in innate immunity and inflammation. These NLRs display a central nucleotide-binding domain, an N-terminal protein interaction domain, and a C-terminal leucine-rich repeat (LRR) domain (7). Two prominent members of NLRs are NOD1 and NOD2, whose recognition motifs in bacterial peptidoglycan have been mapped. The minimal peptidoglycan structure that NOD1 recognizes is a dipeptide, γ-D-Glu-meso-diaminopimelic acid (iE-DAP) (8) or a tripeptide, L-Ala-γ-D-Glu-meso-diaminopimelic acid (9) derived mostly from gram-negative bacteria , whereas NOD2 recognizes the minimal peptidoglycan muramyl dipeptide, MurNAc-L-Ala-D-isoGln (MDP), from both gram-positive and gram-negative bacteria (10, 11). Recognition of the ligand leads to NOD1 or NOD2 oligomerization through the central domain and triggers the interaction of their N-terminal caspase-activating and recruitment domain (CARD) with another CARD domain from the downstream adapter molecule receptor-interacting protein 2 (Rip2, also called RICK/CARDIAK) (12). Rip2 activation leads to ubiquitination and degradation of IκB kinase (IKK) γ, the regulator of the IKK complex (13, 14), and Rip2 itself (15, 16), resulting in activation of IKK complex and NF-κB activation. In addition, activation of NOD also leads to activation of MAPK pathways (17-19), although the molecular links between them are less well characterized. Triggering of the signaling pathways leads to proinflammatory cytokine and chemokine gene expression and other host defense response (20).

It has been reported that various TLRs, NOD1 and NOD2 are expressed in adipocytes and adipose tissues of mice and human origin (21-23). Activation of TLR4 or NOD1 leads to proinflammatory responses and insulin resistance in adipocytes (23, 24). Moreover, the proinflammatory environment induced by activation of TLR4 or TLR2 leads to suppression of adipocyte differentiation (25, 26).

We have recently reported the role of NOD1 activation by the synthetic ligand in inducing proinflammatory cytokine/chemokine expression and insulin resistance in adipocytes (23). Both NOD1 and NOD2 mRNA were markedly up-regulated upon differentiation of 3T3-L1 and human primary preadipocyte cultures derived from subcutaneous fat. However, the effects of NOD1 and NOD2 activation on adipocyte differentiation have not been studied.

Here we report that the effects of NOD activation on adipocyte differentiation. Activation of NOD1, but not NOD2, suppressed 3T3-L1 adipocyte differentiation; in contrast, activation of NOD1 and NOD2 both suppressed adipocyte differentiation of human adipose-derived adult stem cells.

METHODS AND PROCEDURES

Reagents

NOD1 synthetic ligand C12-iEDAP and NOD2 synthetic ligand MDP were purchased from invivoGen (San Diego, CA). LPS from E. coli 055:B5 (L2880),

methylisobutylxanthine, dexamethasone, rosiglitazone and insulin were purchased from Sigma (St. Louis, MO).

Cell culture and induction of adipocyte differentiation

Murine 3T3-L1 fibroblasts (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Hyclone) in 5% CO2, 37°C environment until they reach confluence. The differentiation was initiated as described (27). Briefly, on the day the cells reach confluence (designated as day 0, D0), cells were treated with differentiation DMEM containing 10% Fetal bovine serum (FBS, Atlas biologicals), 10µg/mL insulin, 1µM dexamethasone, and 0.5mM 3-isobutyl-1-methylxanthine for 3 days. The cells were then grown in maintenance DMEM containing 10% FBS and 10µg/mL insulin for an additional two days followed by DMEM containing 10% FBS on day 5 (D5) until the cells were fully differentiated on day 7 (D7). Human adipose-derived adult stem cells were purchased from Zen-bio (Research Triangle Park, NC) and were grown and differentiated according to the manufacturer's instructions. Briefly, the cells were seeded and grown in 60mm tissue culture dishes in preadipocyte medium until confluence. The differentiation was initiated with adipocyte differentiation medium for 7 days and maintained in adipocyte maintenance medium for additional 7 days. All media used for human primary cell culture were purchased from Zen-bio.

To study the effects of NOD activation on differentiation, the cells were differentiated in the presence or absence of NOD1 ligand C12-iEDAP, NOD2 ligand MDP, TLR4 ligand LPS or cytokine TNF-α during the whole process.

3T3-L1CARΔ1 cells, which stably express coxsackie and adenovirus receptor, was provided by Dr. David J. Orlicky (University of Colorado Health Sciences Center) (28). As previously described (29), this cell line with stable expression of the truncated receptor for coxsackievirus and adenovirus receptor (CAR) has ~100-fold greater adenoviral infection efficiency, yet with similar insulin response compared to parental 3T3-L1 cells. 3T3-L1CAR Δ1 cells were grown and maintained as described (29).

NF-κB reporter gene assay, transient transfection, and adenovirus transduction

3T3-L1CARΔ1 cells or hADSCs were seeded in 24-well plates and were transduced with adenovirus expressing NF-κB-Luc reporter gene or β-galactosidase (β-gal) for 24 hr. The cells were treated with C12-iEDAP, MDP, LPS or vehicle control for 15hr before the cells were lysed and reporter luciferase activities were measured with GloMax®-Multi+ Detection System (Promega). The luciferase activities were normalized by β-gal activities.

3T3-L1CARΔ1 cells were seeded and transfected with PPRE-Luc reporter gene (Addgene, Cambridge MA) using Fugene HD transfection reagent (Promega, Madison, WI) for 24 hr. The cells were either pretreated with C12-iEDAP or LPS followed by co-treatment with PPARγ synthetic ligand rosiglitazone for 15 hr before lysis or further

transduced with adenovirus expressing IκBα super-repressor (30) or β-gal for additional 24 hr before the treatment and lysis. Reporter luciferase activities were measured and normalized by protein concentrations.

Oil Red O staining and quantification

To quantify lipid accumulation, differentiated cells were fixed with 4% paraformaldehyde overnight, then rinsed with deionized water and stained with Oil Red O solution (60% Oil Red O in isopropanol) for 10 min. After staining, cells were rinsed with deionized water and cell pictures were taken. To quantify the staining, the Oil Red O was eluted using 100% isopropanol for 10 min and measured by OD (absorbance) reading at 500 nm in a spectrophotometer.

RNA preparation and quantitative real-time PCR analysis

At indicated times, total RNA was prepared from adipocytes using Trizol (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. Total RNA abundance was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was carried out using High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA) according the manufacturer's instructions. mRNA expression of various adipocyte marker genes, and loading control 36B4 were measured quantitatively using gene-specific TaqMan gene expression assays (Applied Biosystems) and were run in a 96-well format using an ABI

7300HT instrument. Cycle conditions were 50°C 2 min, 95°C 15 min, then 40 cycles of 95°C for 15 s/60°C for 1 min.

Small RNA interference

3T3-L1 preadipocytes were stably transfected with ready-made psiRNA expressing shRNA targeting mouse NOD1 or negative control gene luciferase (Invivogen, San Diego, CA). si-mNOD1 sequence: GTGAGGAACTGACCAAGTATA; si-Luc sequence: GACTTACGCTGAGTACTTCGA. Stable individual clones were selected and maintained in zeocin-containing media. NOD1 stable knockdown or negative control clones were screened for basal NOD1 mRNA expression and differentiation capability.

Statistical Analysis

All data were presented as means ± SE. Each experiment was repeated at least 3 times. Within an experiment, measurements were performed in triplicates. Data were log transformed when appropriate. Statistical analysis was performed using SigmaPlot 11.0 (Systat Software, Inc.). One way ANOVA with repeated measures were performed followed by multiple comparisons test (Student-Newman-Keuls Method) to determine the differences between the treatment groups or time points. The level of significance was set at $P < 0.05$.

RESULTS

NOD1 activation by the synthetic ligand C12-iEDAP suppressed 3T3-L1 adipocyte differentiation.

We examined the effects of NOD1 activation on 3T3-L1 adipocyte differentiation by treating 3T3-L1 preadipocytes from initiation (D0) to the end of differentiation process (D7) with or without NOD1 ligand C12-iEDAP. The levels of differentiation were assessed by presence of adipocyte morphology, lipid accumulation, and mRNA expression of adipocyte marker genes. NOD1 activation via C12-iEDAP (10 μg/ml) suppressed 3T3-L1 adipocyte differentiation compared to the control as shown by the Oil Red O stained adipocyte morphology (Figure 1a). The overall differentiation and lipid accumulation as judged by Oil Red O absorbance was suppressed to ~40% of the vehicle control ($p<0.01$), which was more potent than LPS with the same concentration (10 μg/ml) (Figure 1a and b). Analysis of mRNA expression of marker genes supported the results of cell morphology and Oil Red O staining (Figure 1c). NOD1 activation suppressed mRNA expression of the two master regulators of adipocyte differentiation: PPARγ and C/EBPα starting from D5 and reached the maximal suppression by C12-iEDAP at D7 ($p<0.01$). Consequently, the adipocyte marker genes involved in lipid metabolism SCD and FABP4 were also suppressed by C12-iEDAP at D3 and/or D5 with the maximal suppression at D7 ($p<0.01$). Similarly, the adipokine adiponectin mRNA was also suppressed by C12-iEDAP at D7 ($p<0.01$). While leptin mRNA was increased by LPS at D7, the differentiated stage, consistent with the report (25), but was suppressed by C12-iEDAP ($p<0.01$) (Figure 1c).

To confirm the effects of C12-iEDAP, we performed dose response study of C12-iEDAP on adipocyte differentiation. C12-iEDAP dose-dependently suppressed mRNA expression of all the adipocyte genes examined, including leptin (Figure 2) at D7.

Adiponectin and leptin are the two most important adipokines that are secreted primarily by adipocytes and play critical roles in modulating whole body homeostasis (31). It has been reported that proinflammatory stimulants suppress adiponectin but increase leptin expression (32, 33). We further examined the effects of C12-iEDAP in differentiated adipocytes on adiponectin and leptin mRNA expression. C12-iEDAP suppressed both adiponectin and leptin mRNA expression in a time and dose-dependent manner in 3T3-L1 adipocytes (Figure 3a and b). Similar results were observed by another synthetic NOD1 ligand Tri-DAP (data not shown).

Figure 1.1: NOD1 activation by C12-iEDAP suppressed 3T3-L1 adipocytes differentiation. 3T3-L1 cells were differentiated in the presence or absence of C12-iEDAP (10 µg/ml) or LPS 10 µg/ml). Oil Red O staining of cell morphology at Day 7 (D7) (**a**) and quantification of Oil Red O absorbance (**b**) were shown. (**c**) Relative mRNA expression of adipocyte markers at D0 (initiation of differentiation), D3, D5 and D7 were analyzed by quantitative RT-PCR using Taqman gene expression assays. The relative gene expression was normalized to 36B4 gene and expressed as fold of D0 vehicle samples (set at 1). Data are mean±SE (n=3). *, #, indicate significant changes with $p<0.05$ and $p<0.01$, respectively.

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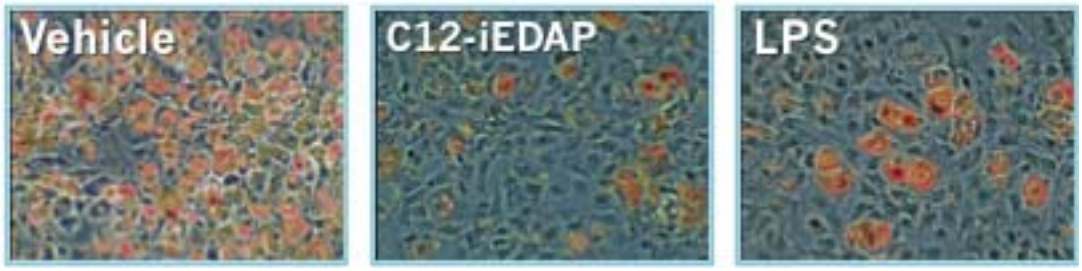
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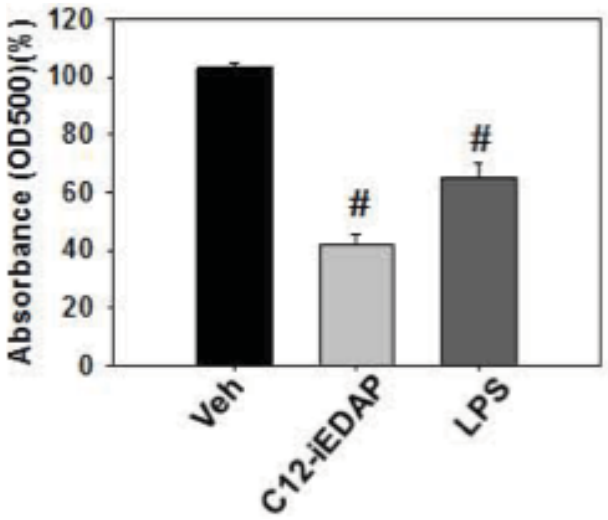
B.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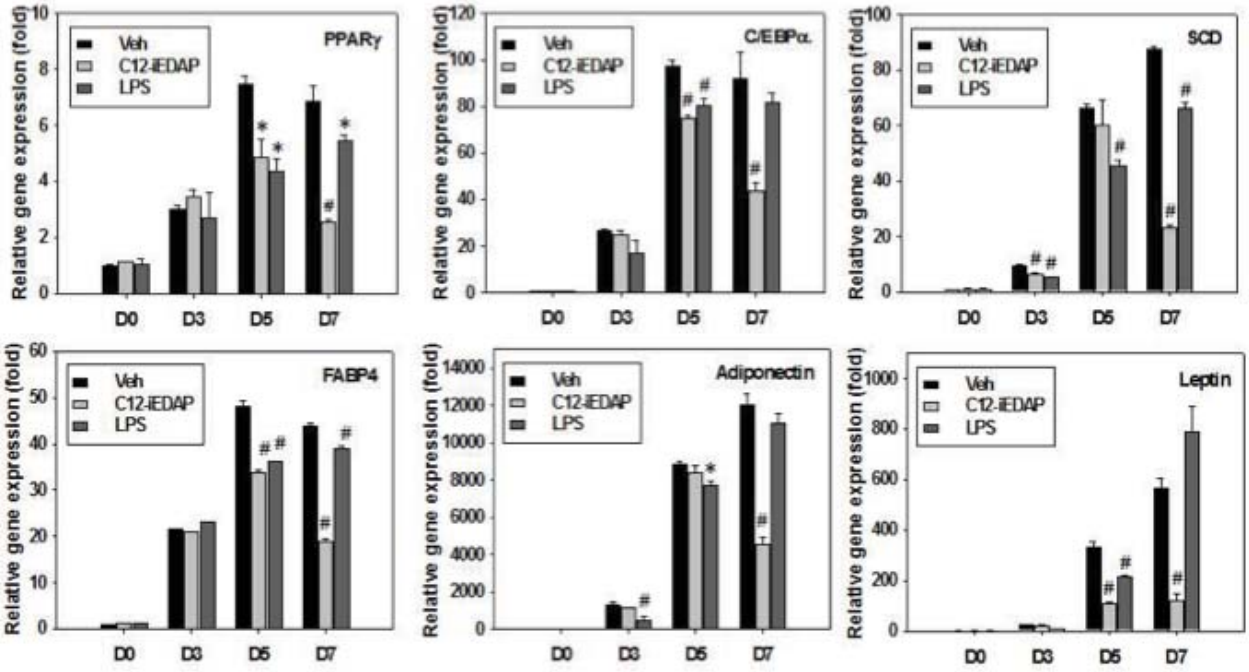
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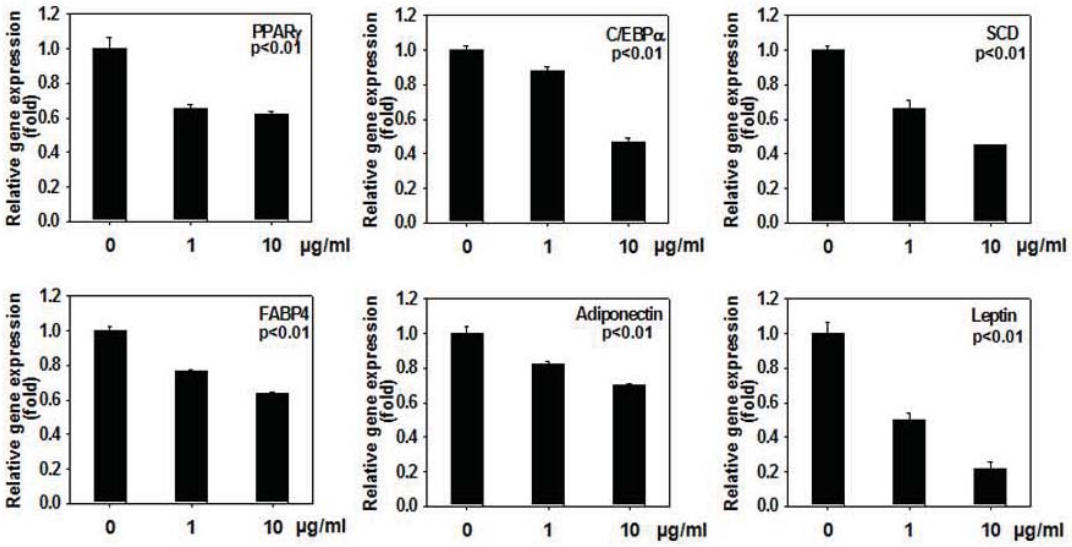


Figure 1.2: The effects of NOD1 activation on 3T3-L1 adipocyte differentiation with increasing doses of C12-iEDAP. 3T3-L1 cells were differentiated in the presence of increasing doses of C12-iEDAP (0, 1, 10 µg/ml). Relative mRNA expression of adipocyte markers at D7 were analyzed by quantitative RT-PCR using Taqman gene expression assays. The relative gene expression was normalized to 36B4 gene and expressed as fold of D0 vehicle samples (set at 1). Data are mean±SE (n=3).

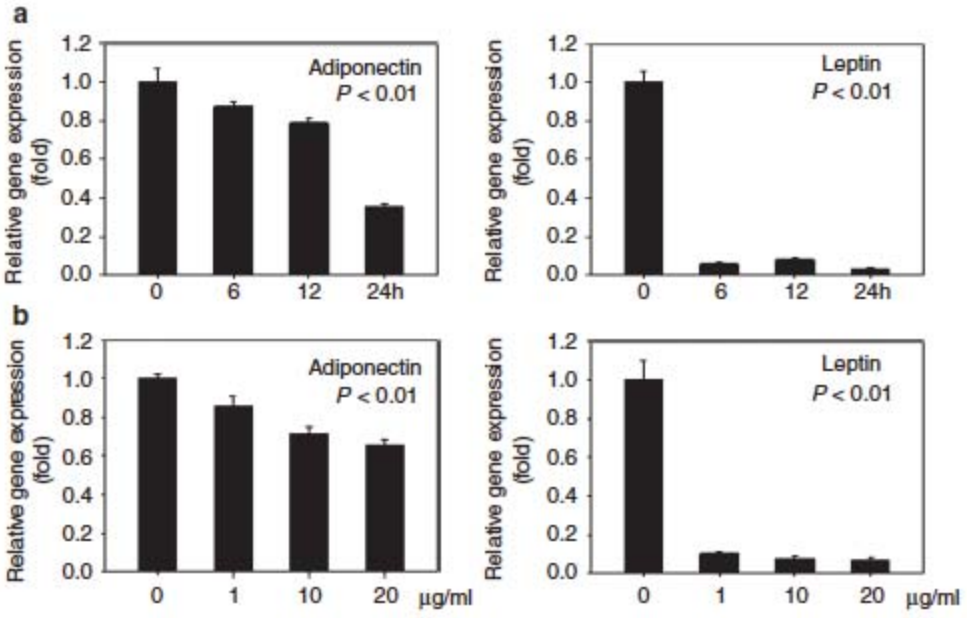


Figure 1.3 NOD1 activation with C12-iEDAP suppressed both adiponectin and leptin mRNA in a time-and dose-dependent manner in differentiated 3T3-L1 adipocytes. 3T3-L1 adipocytes (D7) were treated at indicated times (a) and doses (b) with C12-iEDAP. Relative adiponectin and leptin mRNA were analyzed by quantitative RT-PCR using Taqman gene expression assays. The relative gene expression was normalized to 36B4 gene and expressed as fold of D0 vehicle samples (set at 1). Data are mean±SE (n=3).

NOD2 activation did not suppress 3T3-L1 adipocyte differentiation.

To investigate whether NOD2 activation had similar effects on 3T3-L1 differentiation, we treated the cells with various concentrations of NOD2 ligand, MDP, from D0 to D7. NOD2 activation by MDP up to 100 µg/ml did not suppress differentiation at D7 as judged by Oil Red O stained cell morphology (Figure 4a) and absorbance (Figure 4b). mRNA expression of selected marker genes confirms the lack of suppression on differentiation by NOD2 activation (Figure 4c). Time course analysis revealed that NOD2 activation by MDP at 100 µg/ml only transiently suppressed the differentiation at D3, but the suppression was not maintained through the end of the differentiation process (Figure 4d).

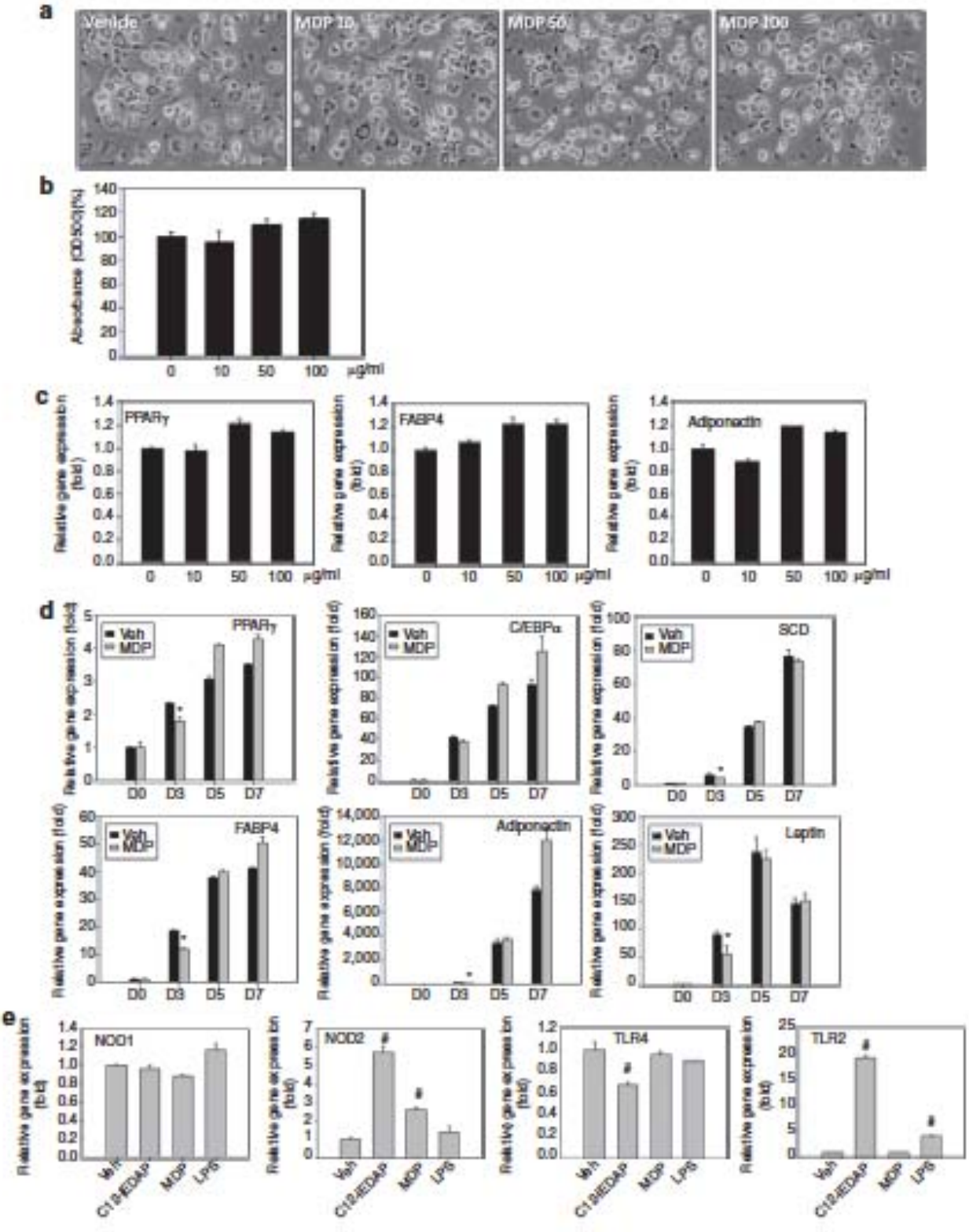
Stimulation of TLR4 with LPS has been shown to increase TLR2 gene expression in 3T3-L1 adipocytes and primary adipocytes from mice (34). We examined whether exposure of NOD1 ligand or NOD2 ligand during the differentiation induces changes in the gene expression of NOD1 and NOD2 as well as TLR4 and TLR2, which could affect differentiation. The most dramatic effects were observed at D7. C12-iEDAP and MDP both significantly induced NOD2 mRNA ($p<0.01$); however, no significant effects on NOD1 mRNA were observed. C12-iEDAP also significantly increased TLR2 mRNA ($p<0.01$), but suppressed TLR4 mRNA ($p<0.01$) (Figure 4e). TLR4 activation by LPS increased TLR2 mRNA in 3T3-L1 cells, as reported (34) (Figure 4e).

Since the activation of NOD1, but not NOD2, suppresses 3T3-L1 adipocyte differentiation, to confirm that the suppression by C12-iEDAP on 3T3-L1 differentiation were via NOD1 in our experiments, we generated individual stable 3T3-L1 clones whose NOD1 mRNA has been stably knocked down by small RNA interference targeting NOD1 (shNOD1) to ~20% of non-transfected control cells (Control) (Figure 5a). Compared with Luc-targeting control cells (shLuc), NOD1 stable knockdown reversed the suppression of C12-iEDAP on differentiation, shown by the PPARγ and C/EBPα mRNA, markers of differentiation (Figure 5), but had no effects on MDP's effects, confirming that the specificity of the effects of NOD1 activation.

It has been reported that proinflammatory cytokine TNFα inhibits adipocyte differentiation through suppression of PPARγ transcriptional activation by TAK1/TAB/NIK kinase-mediated NF-κB activation (35). To study the role of NF-κB in NOD1-mediated suppressive effects on differentiation, we employed 3T3-L1 cells stably transfected with truncated receptor for coxsackievirus and adenovirus receptor (3T3-L1CARΔ1) (28, 29). We confirmed that C12-iEDAP and LPS, but not MDP, activated NF-κB, as determined by NF-κB reporter gene assays ($p<0.01$) (Figure 6a). Moreover, C12-iEDAP and LPS, but not MDP, suppressed the synthetic ligand rosiglitazone-induced PPARγ activation ($p<0.01$ and $p<0.05$, respectively) (Figure 6b). The suppressive effects by C12-iEDAP and LPS were reversed by IκB super-repressor (IκB(SR)), which contains two amino acid substitutions (S32A/S36A) that prevent

phosphorylation and degradation of the protein and blocks the activation of NF-κB in response to proinflammatory stimulations (30) (Figure 6c).

Figure 1.4 NOD2 activation by MDP did not suppress 3T3-L1 differentiation. 3T3-L1 cells were differentiated in the presence of increasing doses of MDP (0, 10, 50, 100µg/ml). Oil Red O staining of cell morphology at Day 7 (D7) (a) and quantification of Oil Red O absorbance (b) were shown. (c) Relative mRNA expression of adipocyte markers at D7 were analyzed. (d) The effects of MDP (100µg/ml) on mRNA expression of adipocyte markers at D0, D3, D5 and D7 were analyzed. (e) The effects of NOD1 and NOD2 activation on mRNA expression of TLR4, TLR2, NOD1 and NOD2. Relative mRNA expression of NOD1, NOD2, TLR4 and TLR2 were analyzed at D7 when 3T3-L1 cells were differentiated in the presence of C12-iEDAP, MDP, LPS or vehicle control. Gene expression was analyzed by quantitative RT-PCR using Taqman gene expression assays, normalized to 36B4 gene and expressed as fold of D0 vehicle samples (set at 1). Data are mean±SE (n=3). *, #, indicate significant changes with $p<0.05$ and $p<0.01$, respectively.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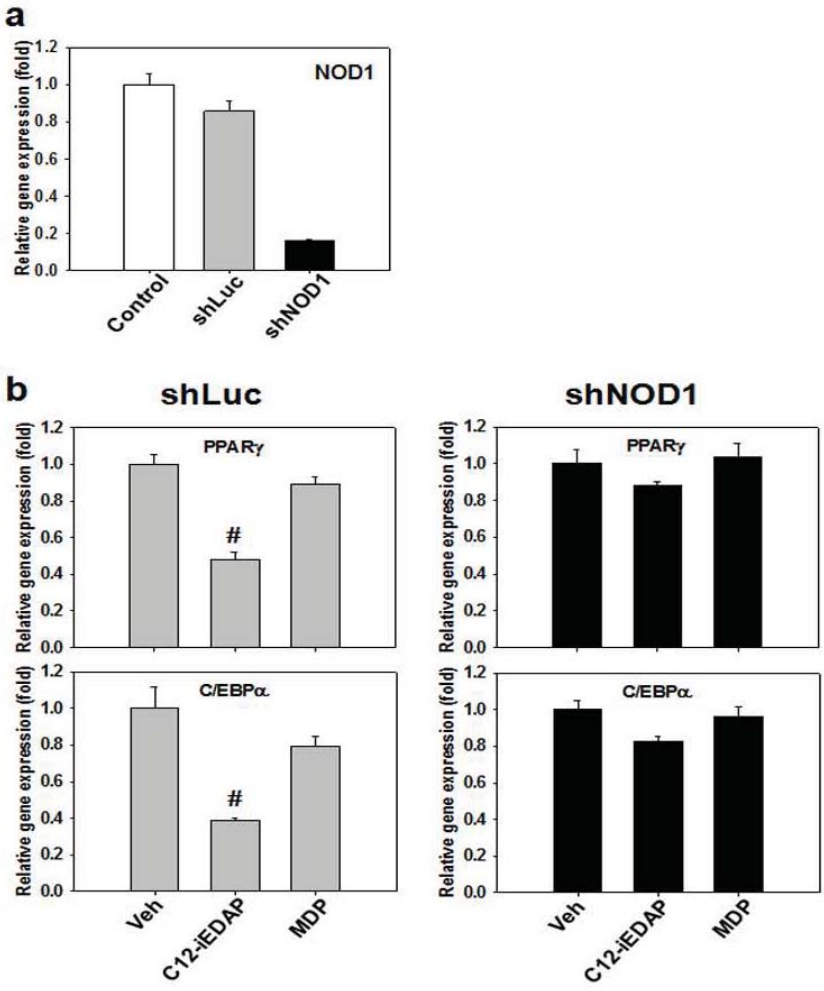


Figure 1.5 The effects of C12-iEDAP on adipocyte differentiation were via NOD1. 3T3-L1 stable knockdown with shRNA targeting NOD1 (shNOD1), non-targeting cells (shLuc) or non-transfected control cells were analyzed for NOD1 mRNA expression (a) and were differentiated in the presence of C12-iEDAP, MDP or vehicle (b). (b) Relative mRNA expression of adipocyte markers at D5 were analyzed by quantitative RT-PCR using Taqman gene expression assays. The relative gene expression was normalized to 36B4 gene and expressed as fold of vehicle treated samples (set at 1). Data are mean±SE (n=3). *, indicate significant changes with $p<0.05$.

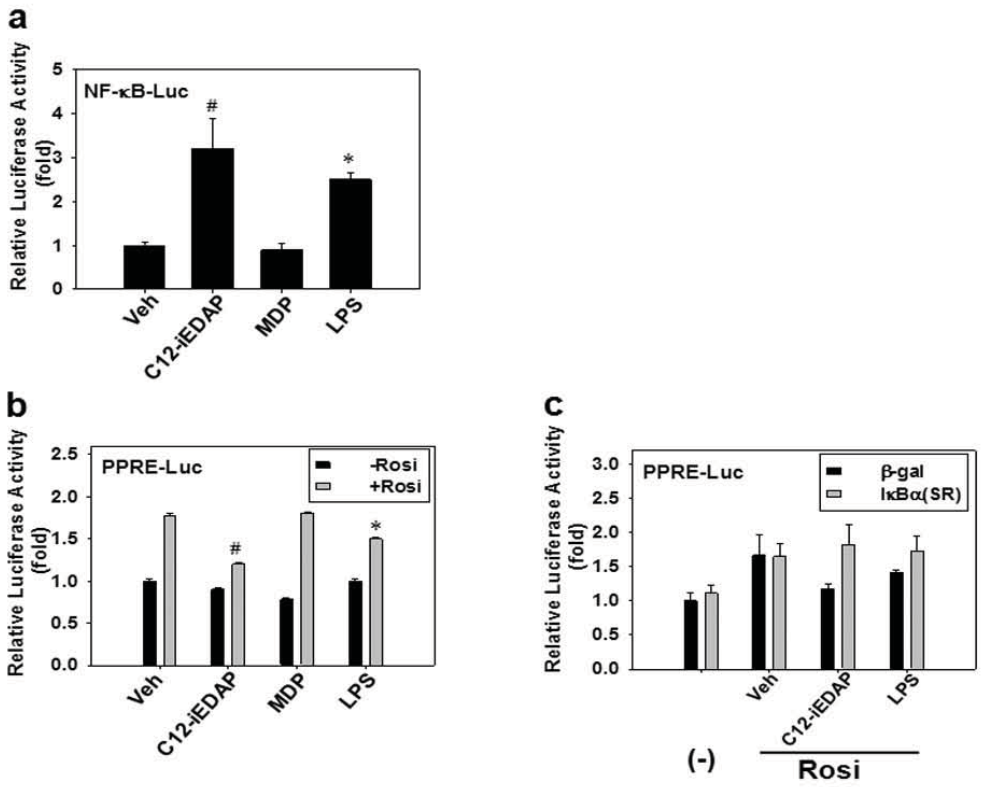
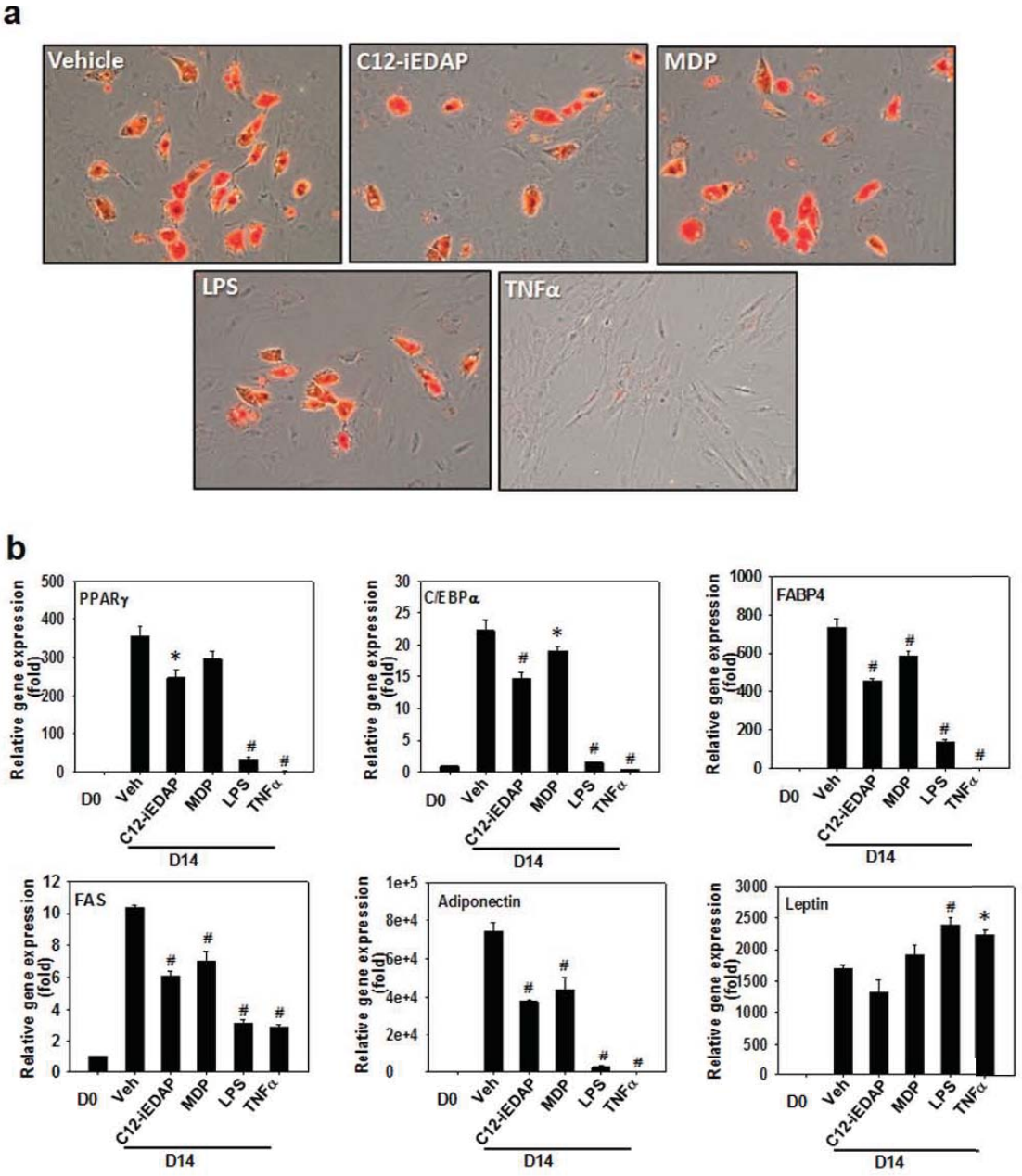


Figure 1.6 NOD1 activation suppressed ligand-induced PPARγ transactivation through NF-κB pathway in 3T3-L1 cells. (a) 3T3-L1CARΔ1 cells were transduced with adenovirus containing with NF-κB-Luc reporter gene for 24hr. The cells were stimulated with C12-iEDAP, LPS, MDP or vehicle for 15hr before lysis. (b) 3T3-L1CARΔ1 cells were transiently transfected with PPRE-Luc for 24hr. The cells were pretreated with C12-iEDAP, LPS or MDP for 1hr followed by co-treatment with PPARγ synthetic ligand rosiglitasone (rosi) for 15hr before lysis. (c) 3T3-L1CARΔ1 cells were transiently transfected with PPRE-Luc for 24hr and were then transduced with adenovirus containing β-gal or IκBα(SR)) for further 24hr. The cells were pretreated with C12-iEDAP, LPS or MDP followed by co-treatment with rosi for 15hr before lysis. The reporter gene assays were performed and data are mean±SE (n=3). *, #, indicate significant changes with $p<0.05$ and $p<0.01$, respectively.

The effects of NOD activation on adipocyte differentiation of human adipose-derived adult stem cells.

We further examined the effects of NOD activation on adipocyte differentiation of human adipose-derived adult stem cells (hADSC). We first examined whether NOD1 and NOD2 can be activated in hADSCs by NF-κB reporter gene assays. Activation of NOD1 ($p<0.01$) and TLR4 ($p<0.01$), but not NOD2, induced NF-κB activation in hADSCs (Figure 7a). Next, hADSCs were differentiated in the presence of either C12-iEDAP, MDP or vehicle control (D0 to D14). In contrast to 3T3-L1 cells, both C12-iEDAP and MDP suppressed the differentiation as judged by Oil Red O stained cell morphology (Figure 7b, and a panel of adipocyte marker genes (Figure 7c). In addition, similar to 3T3-L1 adipocytes, C12-iEDAP suppressed both adiponectin ($p<0.01$) and leptin mRNA ($p=0.06$) at D14. MDP suppressed adiponectin mRNA ($p<0.05$), but had little effect on leptin ($p=0.2$). Both LPS and TNFα suppressed adiponectin mRNA ($p<0.01$) but increased leptin mRNA ($p<0.01$ and $p<0.05$, respectively) in adipocytes differentiated from hADSCs (Figure 7c). We also examined the effects of NOD1 or NOD2 activation on mRNA expression of NOD1 and NOD2 as well as TLR4 and TLR2 in hADSCs at D14. Activation of NOD1 by C12-iEDAP did not significantly affect mRNA expression of NOD1, NOD2, TLR4 and TLR2 in hADSCs (Figure 7d). In contrast, NOD2 activation by MDP only significantly decreased its own mRNA expression ($p<0.05$) (Figure 7d). Similar to 3T3-L1, LPS also significantly increased TLR2 mRNA in hADSCs ($p<0.01$) (Figure 7d).

Figure 1.7 Activation of NOD1 and NOD2 both suppressed adipocyte differentiation of human adipose-derived adult stem cells. hADSCs were differentiated in the presence of C12-iEDAP, MDP, LPS, TNFα or vehicle. Oil Red O stained cell morphologies at Day 14 were shown (a). (b) Relative mRNA expression of adipocyte markers at D0 (initiation of differentiation), and D14 were analyzed by quantitative RT-PCR using Taqman gene expression assays. (c) The effects of NOD1 and NOD2 activation on mRNA expression of TLR4, TLR2, NOD1 and NOD2. Relative mRNA expression of NOD1, NOD2, TLR4 and TLR2 were analyzed at D14 when hADSCs were differentiated in the presence of C12-iEDAP, MDP, LPS or vehicle control. The relative gene expression was normalized to 36B4 gene and expressed as fold of either D0 vehicle or vehicle control (set at 1). (d) hADSCs were transduced with adenovirus containing NF-κB-Luc reporter gene and adenovirus containing β-galactosidase for 24hr. The cells were treated with C12-iEDAP, MDP or LPS for 15hr before lysis. Reporter gene assasys were performed. Data are mean±SE (n=3). *, #, indicate significant changes with $p<0.05$ and $p<0.01$, respectively.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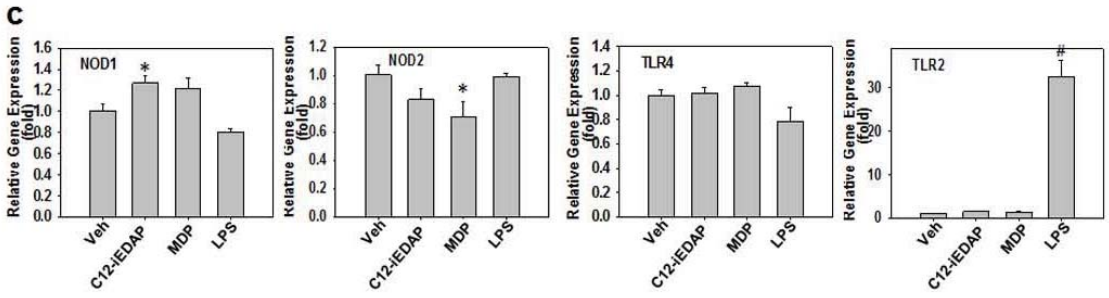
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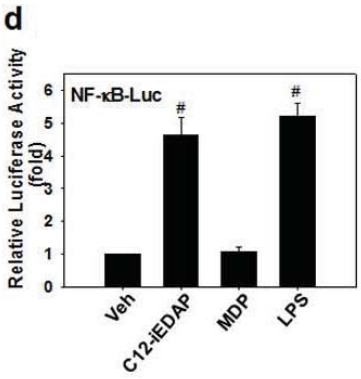
MDP
LPS


d
NF-κB-Luc
Relative Luciferase Activity (fold)
Veh
C12-iEDAP
MDP
LPS

DISCUSSION

Accumulating evidence has shown that obesity is associated with chronic inflammation (3, 4). Two families of pattern recognition receptors, TLR and NLR, NOD1 and NOD2 in particular, have been shown to play critical roles in inflammation in adipocytes (21-23). A proinflammatory environment induced by activation of TLR4 or TLR2, leads to the suppression of adipocyte differentiation (25, 26). Here we show that activation of NOD1, but not NOD2, suppresses 3T3-L1 differentiation, whereas activation of NOD1 and NOD2 similarly suppress the adipocyte differentiation of human adipose-derived adult stem cells.

Our results support the notion that a proinflammatory environment impairs adipocyte differentiation. Interestingly, NOD1 and NOD2 activation also suppress adipocyte differentiation of mesenchymal stem cells derived from human umbilical blood (36). Since inflammation inhibits adipocyte differentiation but promotes endothelial cell differentiation, it has been suggested that as a major influence in adipose tissue microenvironment in the obese state, inflammation could serve as signal mediating the competition between adipocytes and endothelial cells for the limited source of adipose-derived stem cells (ASC), which have the potential to be differentiated into multiple linages of progenitor cells including adipocytes, endothelial cells, fibroblasts (37). By suppressing adipocyte differentiation, NOD activation could favor ASC differentiation into endothelial cells leading to enhanced angiogenesis in adipose tissue. Further

studies are needed to elucidate the role of NOD activation in adipose inflammation in obesity.

NOD1 and NOD2 activation also affect adipokine expression during and/or post adipocyte differentiation. Similar to TLR4 activation and TNFα, activation of NOD1 and NOD2 (in hADSCs) suppressed adiponectin mRNA expression. In addition, NOD1 activation also suppressed leptin mRNA. Our result that NOD1 activation suppresses rather than increases leptin mRNA is in contrast to the notion that proinflammatory stimulants (e.g., LPS or TNFα) increase leptin expression (32). Leptin is a pleiotropic molecule that regulates not only food intake and metabolic and endocrine functions, but also immunity, inflammation and hematopoiesis (31, 32). The most well known role for leptin is the regulation of appetite, as either the absence of leptin or a mutation in leptin receptor genes induces a massive hyperphagia and obesity in animal models and humans (32). Leptin deficiency causes dysregulation of the immune and inflammatory response observed in the animals with absence of leptin or mutation of leptin receptor (32). Moreover, leptin levels are also increased by inflammatory stimulants, such as LPS and TNFα, in experimental animals (32). It has been suggested that increase in leptin expression may mediate the anorexia of inflammation (38). The fact that NOD1 activation suppresses rather than increases leptin expression suggests the differential effects of NOD1-mediated inflammation in adipocytes, compared to TLR4 and the classical cytokine TNFα. The role of NOD1 activation in adipose inflammation and whole body homeostasis needs to be elucidated further.

The species specific effects of NOD activation on adipocyte differentiation have been noted. While NOD1 activation suppressed adipocyte differentiation of both 3T3-L1 and hADSCs, NOD2 activation only suppressed adipocyte differentiation of hADSCs. The differential effects may be due to the differences in NOD2 expression levels between murine 3T3-L1 and human ADSCs. We have found that the relative NOD2 mRNA expression (relative to 18S) in hADSCs was more than twofold of that of 3T3-L1 cells (data not shown), which may render hADSCs more sensitive to NOD2 ligand MDP. In addition, we showed C12-iEDAP induced robust up-regulation of NOD2 and TLR2 mRNA in 3T3-L1 cells but had minimal effects on mRNA expression of these receptors in hADSCs. In contrast, MDP induced up-regulation of NOD2 mRNA in 3T3-L1, but suppressed it in hADSCs. These results further demonstrate the species specific cellular responses upon NOD activation in adipocytes.

The mechanisms underlying the effects of inflammation on adipocyte differentiation have been suggested (37). It has been shown that TNFα suppresses adipocyte differentiation by suppressing the master transcriptional factor PPARγ mRNA and transcriptional activity through NF-κB pathway. We showed that NOD1 activation by C12-iEDAP suppressed mRNA of PPARγ and C/EBPα in both 3T3-L1 and hADSCs. NOD2 activation by MDP also suppressed mRNA expression of these two transcriptional factors in hADSCs; however, MDP only transiently suppressed mRNA of these two transcriptional factors at day 3 in 3T3-L1 cells, which did not result in

suppression of differentiation in the end. Moreover, we confirmed that C12-iEDAP and LPS, but not MDP, induced NF-κB activation as determined by NF-κB reporter gene assays, which were correlated with their abilities to suppress ligand-induced PPARγ transactivation in 3T3-L1 cells. We further demonstrated that the suppressive effects by C12-iEDAP and LPS on PPARγ transactivation were reversed by IκB super-repressor which blocks IκB degradation and NF-κB activation. Together, these results demonstrate that NOD1 activation, similar to TLR4 activation, suppresses PPARγ activity (mRNA and transactivation) in 3T3-L1 cells. Moreover, inflammation could affect adipocyte differentiation through suppressing insulin signaling in adipocytes. We have reported that NOD1 activation led to impaired insulin signaling in adipocytes (23). Therefore, it is conceivable that NOD1 activation could suppress adipocyte differentiation through suppression of insulin signaling. Furthermore, NOD1 activation by C12-iEDAP induced mRNA expression of NOD2 and TLR2, but decreased TLR4 mRNA; therefore, NOD1 activation could suppress differentiation by modulating the activation of other PRRs (e.g., TLR2 and NOD2) through modulating their mRNA expression. Further studies are needed to elucidate the molecular mechanisms underlying NOD1-mediated effects on adipocyte differentiation.

Interestingly, even though NOD2 activation by MDP suppressed adipocyte differentiation, it did not activate NF-κB, as determined by NF-κB reporter gene assays, in hADSCs. Therefore, the mechanisms by which MDP suppressed adipocyte differentiation of hADSCs need to be explored further. It has been reported that

activation of NOD2 as well as NOD1 induced autophagic response to invasive bacteria, independent of NF-κB activation (39), suggesting that NOD2 can mediate cellular response that are independent of NF-κB pathway. Moreover, autophagy, a process that is responsible for the clearance of damaged or old organelles, and large protein aggregates in the cytosol, has been shown to regulate adipose mass, adipocyte differentiation and the balance between white and brown fat in mice (40). It is possible that MDP could induce NOD2-mediated autophagy thereby affecting adipocyte differentiation in hADSCs. Further studies are needed to unravel the mechanisms underlying the effects of NOD2 activation on adipocyte differentiation and whether NOD-mediated autophagy is involved in the process.

Overall, our results suggest that NOD1 and NOD2 may represent novel targets for adipose inflammation in obesity. Understanding the role of NOD proteins in adipose tissue and whole body homeostasis may provide novel strategies for obesity prevention and treatment.

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CHAPTER II

ACTIVATION OF NUCLEOTIDE-OLIGOMERIZATION DOMAIN PROTEIN 1 INDUCES LIPOLYSIS THROUGH NF-κB AND THE LIPOLYTIC PKA/HSL PATHWAYS IN 3T3-L1 ADIPOCYTES

A version of the following manuscript will be published Jaanki S Purohit, Pan Hu, Guoxun Chen, Jay Whelan, Naima Moustaid-Moussa, and Ling Zhao (corresponding author) under the title, “Activation of Nucleotide-Oligomerization Domain Containing Protein 1 Induces Lipolysis Through NF-κB and the Lipolytic PKA Activation in 3T3-L1 Adipocytes” in the journal *Biochemistry and Cell Biology* (2013).

ABSTRACT

Obesity is associated with chronic inflammation. Toll-like receptors (TLR) and NOD-like receptors (NLR) are two families of pattern recognition receptors that play important roles in the immune response and inflammation in adipocytes. Activation of TLR4 has been shown to stimulate lipolysis from adipose tissue in vivo and from primary adipocytes in vitro, contributing to dyslipidemia associated with endotoxin. However, the effects of NOD1 activation on adipocyte lipolysis have not been studied. Here we show that NOD1 activation by the synthetic ligands (Tri-DAP and C12-iEDAP) stimulate basal lipolysis in 3T3-L1 adipocytes in a time and dose dependent manner. The effect of C12-iEDAP on lipolysis is attenuated with NOD1 siRNA knockdown, demonstrating the specificity of the effects. Moreover, inhibition of NF-κB pathway, but not MAPK pathways, by pharmacological inhibitors, attenuates the lipolytic effects of C12-iEDAP. Also, inhibition of the PKA/HSL lipolytic pathway also suppresses the lipolytic effects of C12-iEDAP. Furthermore, we show NOD1 activation suppresses protein expression of perilipin, which was mediated through p38 MAPK, but not NF-κB pathway. Taken together, our results demonstrate a novel role of NOD1 activation in adipocytes and suggest that NOD1 activation may contribute to dyslipidemia via enhanced lipolysis.

ABBREVIATIONS

WAT, white adipose tissue; LD, lipid droplet; ATGL, adipose triglyceride lipase; FFA, free fatty acid; HSL hormone sensitive lipase; PLIN, perilipin; Tri-DAP, L-Ala-gamma-D-Glu-mDAP; C12-iEDAP, Lauroyl-γ-D-glutamyl-meso-diaminopimelic acid; LPS, lipopolysaccharide; MGL, monoglyceride lipase;NOD1, nucleotide-binding oligomerization domain-1; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; TLR4, toll-like Receptor 4; NF-κB, nuclear factor kappaB; MAPK, mitogen activated protein kinase; ERK, extracellular signal-related kinase.

INTRODUCTION

It is now generally accepted that obesity is associated with chronic inflammation, which may contribute to obesity related metabolic complications, such as insulin resistance and type 2 diabetes. Chronic inflammation in white adipose tissue (WAT) is thought to affect systemic metabolic homeostasis through elevated secretion of proinflammatory cytokines and/or chemokines (1-3) and free fatty acids (FFA) (4). As its primary role, WAT accumulates energy in the form of triacylglycerols (TAG) in the lipid droplet (LD). In times of metabolic need, TAGs break down into glycerol and free fatty acids (FFA) to be used by other tissues, a process known as lipolysis. FFAs are not only an energy source, they also function as signaling molecules (5). Chronically elevated FFA alters glucose and lipid metabolism in skeletal muscle and liver and may lead to insulin resistance (4).

The regulation of lipolysis is complex and requires multiple enzymes and associated proteins. In times of metabolic need, beta-adrenergic stimulation of the hormone adrenaline and/or neurotransmitter noradrenaline activate protein kinase A (PKA), which in turn phosphorylate and activate hormone sensitive lipase (HSL) to increase the intrinsic activity and promote the translocation of a predominantly cytosolic HSL to LD (6-8). In addition to this well known lipolytic pathway, another TAG lipase, adipose TAG lipase (ATGL) has been recently identified and catalyzes the removal of the sn-1 fatty acid (FA) from TAG and generation of diacylglycerol (9-11), which can be

further hydrolysed by HSL producing monoacylglycerol, with subsequent hydrolysis by the monoacylglycerol lipase (MGL) (12).

It has been demonstrated that LD associated proteins, such as perilipin (PLIN), play important regulatory roles in the process of lipolysis (13). As the most abundant lipid droplet coating protein, perilipin forms a physical barrier to control the access of lipases to LD (14). It has been reported the PLIN-null mice have smaller adipocytes, have elevated basal lipolysis, and are resistant to diet-induced obesity (15).

Innate Immune Function of Adipose Tissue

Pattern recognition receptors (PRRs) are innate immune receptors that recognize pathogen associated molecular patterns (PAMPs) on invading pathogens and mediate the inflammatory response. Accumulating evidence supports the critical role of PRRs in mediating inflammation in adipose tissue and adipocytes in obesity (16). The most well studied PRRs in adipose tissue and adipocytes is toll-like receptor 4 (TLR4). Activation of TLR4 by gram-negative bacteria lipopolysaccharide (LPS) induced proinflammatory cytokine/chemokine expression in adipocytes and insulin resistance (17-19). Also, TLR4 activation by LPS induced enhanced lipolysis, contributing to endotoxin-induced dyslipidemia (20, 21).

We have reported the role of NOD1 activation by synthetic ligands in inducing proinflammatory cytokine/chemokine expression and insulin resistance in adipocytes via

activation of NF-κB and MAPK pathways (22). In addition, NOD1 activation suppresses both 3T3-L1 and human primary adipocyte differentiation as evidenced by lipid accumulation and dose and time-dependent down regulation of differentiation genes (3). However, the effect of NOD1 activation on adipocyte lipolysis has not been studied.

NF- κB activation also leads to the phosphorylation of NF- κB p65 by PKAc (23). Stimulating cells with NF- κB activators such as LPS leads to the degradation of IκB-α and IκB-β proteins, subsequent translocation of NF- κB to the nucleus and activating transcription.

Here, we show that NOD1 activation by the synthetic ligands leads to increased lipolysis in a time and dose dependent manner in 3T3-L1 mature adipocytes. We further explore the involvement of NF-κB and MAPK pathways and possible molecular mechanisms underlying the increased lipolysis by NOD1 activation.

METHODS AND PROCEDURES

Reagents

NOD1 synthetic ligands C12-iEDAP and Tri-DAP were purchased from invivoGen (San Diego, CA). Lipopolysaccharide (LPS), Methylisobutylxanthine, dexamethasone and insulin were purchased from Sigma (St. Louis, MO). Pharmacological inhibitors for NF-κB Caffeic acid phenethyl ester and Bay117821 and the inhibitors for MAPK (SB203580, SP600125, and PD98054) and Protein Kinase A inhibitor (H-89) were

purchased from Tocris Bioscience (Ellisville, MI). A third NF-κB inhibitor, QNZ (CAY10470) and hormone sensitive lipase inhibitor CAY10499 were purchased from Cayman Chemical (Ann Arbor, MI). Antibodies for HSL, ATGL, and PLIN were from Cell Signaling (Boston, MA). Antibodies for MGL (Santa Cruz), monoclonal anti- β-actin (Sigma).

Cell culture and treatment

Murine 3T3-L1 fibroblasts (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Hyclone) in 5% CO2, 37°C environment until they reached confluence. The differentiation was initiated as described (22, 24) in 6-well and 96-well plates. Briefly, on the day the cells reach confluence (designated as day 0, D0), cells were differentiated with DMEM containing 10% fetal bovine serum (FBS, Atlas biological, Fort Collins, CO), 10 µg/mL insulin, 1 µM dexamethasone, and 0.5mM 3-isobutyl-1-methylxanthine for 3 days, followed by DMEM containing 10% FBS and 10 µg/mL insulin for an additional two days. The cells were then maintained in DMEM containing 10% FBS until stimulated. Typically, adipocyte conversion occurred in 99% of the cells 8 days post-initiation of differentiation (D8). To induce the basal lipolysis, adipocytes at D8 were washed with wash buffer (x2) provided by the lipolysis kit, and cells were then incubated in assay buffer in the presence or absence of NOD1 ligands (Tri-DAP and C12-iEDAP) or TLR4 ligand (LPS) at 37°C in a

humidified incubator for indicated periods of time. The conditioned media was collected at the end of the incubation and analyzed for glycerol content or free fatty acid content as indicated. To test the effects of pharmacological inhibitors, the cells were pretreated with the inhibitors for 1 hr and then co-treated with the ligands for the entire duration of the incubation.

Lipolysis and free fatty acid detection

Glycerol or free fatty acids released into the cellular supernatants from triglyceride breakdown was determined using Adipocyte Lipolysis Assay kit for 3T3-L1 cells (Zen-Bio, Research Triangle Park, NC). Briefly, Cells were differentiated in 96-well plates. At D9, fully differentiated adipocytes were washed with PBS (x3) before replacing with Assay Buffer. Cells were incubated in the assay buffer in the presence or absence of the ligands in triplicates for 24 hrs. To measure glycerol or fatty acid release, 100 µl of the conditioned assay buffer from each set of triplicates was collected at the end of 24 hrs in a separate 96-well plate for detection using colorimetric absorbance according to the manufacturer's instructions.

Western blot analysis

The protein concentrations were determined by BCA assay kit (Thermo Scientific, Waltham, MA). Total cell lysates (10-40 µg) were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The

membrane was blocked in 20 mM Tris–HCl, 137 mM NaCl, and 0.1% (v/v) Tween 20 (pH 7.4) containing 5% non-fat milk. The membrane was immunoblotted with primary antibodies for 1–24 hr, followed by secondary antibody coupled to horseradish peroxidase (GE Healthcare, Piscataway, NJ) for 1 hr. The membrane was exposed on an X-ray film using ECL Western blot detection reagents (GE Healthcare). To reprobe with different antibodies, the membrane was stripped in stripping buffer containing 62.5 mM Tris–HCl, 2% SDS and 100 mM 2-mercaptoethanol at 50 °C for 20-30 min. The signal was quantified by densitometry using ChemiDocXRS+ imaging system with image lab software (Bio-Rad).

Small RNA interference

Small RNA interference against NOD1 or non-targeting controls was performed in differentiated 3T3-L1 adipocytes. siRNA oligo targeting against murine NOD1 was from *Silencer Select* from Ambion and were transfected into 3T3-L1 adipocytes with DeliverX plus system (Panomics) according to the manufacturer's protocol.

PKA and cAMP Activation

Activation of PKA and cAMP was measured using DetetX® kits (Arbor Assays, MI). PKA activation was detected by lysing fully differentiated adipocytes in Activated Cell lysis buffer. Lysed cells were centrifuged at 10,000 rpm for 10 min and supernatant was aspirated for analysis. Supernatant was diluted 1:10 into prepared Kinase Assay Buffer prior to running the assay. Diluted supernatant and zero standard samples were

prepared in duplicates in a 96-well plate. 10 µl of reconstituted ATP was added to each well and then incubated at 30°C shaking for 90 min. After incubation, wells were washed 4 times and 25 µL of Goat anti-rabbit IgG HRP conjugate was added to each well. Then 25 µL of Rabbit Phospho PKA substrate was added to each well—cells were incubated for 60 min and washed 4 times. 100 µL of TMB substrate solution was added to each well. The plate was incubated for 30 min, stop solution was added, and the optical density was read at 450 nm.

Similarly, cAMP was read using a 25 µL of plate primer to add to the sample and diluent. cAMP conjugate and cAMP antibody were added to each well and incubated for 2 hours. After washing 4 times, 100 µL of TMB substrate was added to each well and incubated for 30 min. Plate was read at 450 nm after adding Stop Solution to each well.

Statistical Analysis

All data were presented as means ± SE. Each experiment was repeated at least 3 times. Within an experiment, measurements were performed in triplicates. Data were log transformed when appropriate. Statistical analysis was performed using SigmaPlot 11.0 (Systat Software, Inc.). One way ANOVA with repeated measures were performed followed by multiple comparisons test (Student-Newman-Keuls Method) to determine the differences between the treatment groups or time points. The level of significance was set at $P < 0.05$.

RESULTS

NOD1 activation by the synthetic ligand induces lipolysis in 3T3-L1 adipocytes

We examined the lipolytic effects by NOD1 activation in mature 3T3-L1 adipocytes using two different synthetic ligands: Tri-DAP and C12-iEDAP. The cells were treated with each ligand for 6, 12 and 24 hr. The glycerol released into the supernatant was analyzed as a measure for TAG hydrolysis. Both NOD1 ligands induced significant glycerol release when the cells were treated for 24 hr ($p<0.05$) (Fig.1A). The effect of C12-iEDAP on glycerol release was dose-dependent and reached the highest induction at 10 µg/ml ($p<0.001$). TLR4 ligand LPS also induced glycerol release, consistent with previous reports (20, 21). Isoproterenol (ISO), a non-specific -adrenergic agonist, was used as the positive control for lipolysis. DMSO was included as a vehicle control for the isoproterenol. To confirm the glycerol release was from TAG breakdown, we also measured the FFA release from TAG breakdown in the supernatant. NOD1 ligand C12-iEDAP also dose-dependently induced FFA release with the highest induction observed at 10 µg/ml (Fig. 1B). Therefore, we have chosen to use C12-iEDAP at 10 µg/ml for the subsequent experiments.

To test the specificity of the effects of C12-iEDAP on lipolysis was mediated through NOD1, we performed siRNA knockdown targeting against NOD1 in adipocytes. We obtained ~50% knockdown of NOD1 mRNA in D9 mature adipocytes (Fig. 2A), and

NOD1 knockdown attenuated C12-iEDAP-induced lipolysis in 3T3-L1 adipocytes by ~40% (Fig. 2B) ($p<0.001$), demonstrating the specific effect of NOD1 activation on lipolysis.

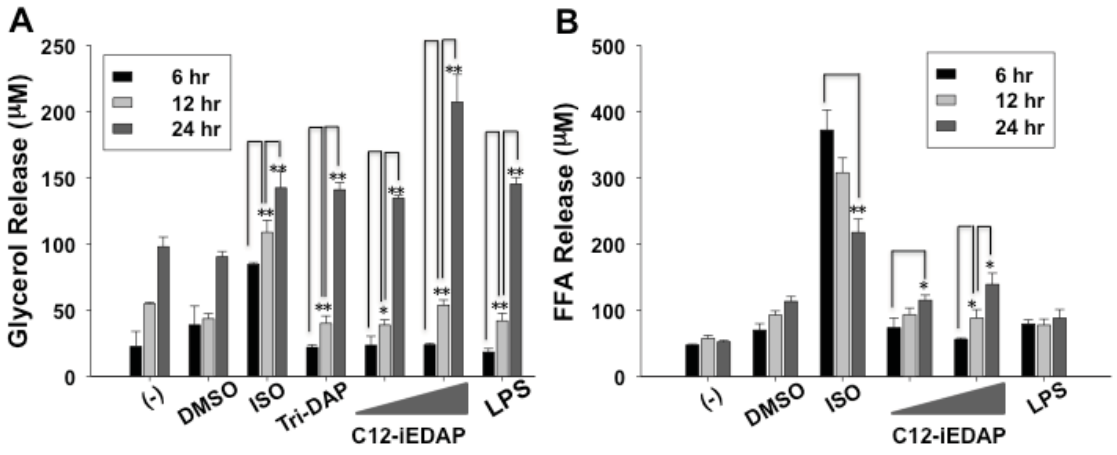

Figure 2.1 NOD1 activation by the synthetic ligands induces lipolysis in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with or without C12-iEDAP (1, 10μg/ml), Tri-DAP (10μg/ml), LPS (10μg/ml), or the positive control isoproterenol (ISO, 1μM) for 6, 12 and 24 hr. The release of glycerol (A) and FFA (B) was measured as μM in the assay buffer; Data are mean±SE (n=3). *, **, indicate significant changes compared to control with $p<0.05$ and $p<0.01$, respectively.

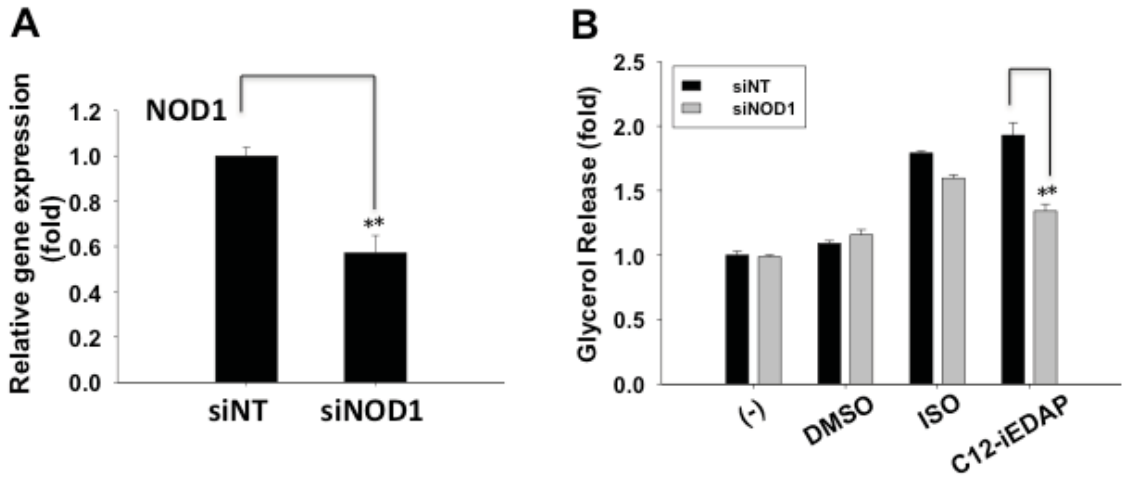

Figure 2.2 C12-iEDAP-induced lipolysis acts specifically through NOD1. 3T3-L1 adipocytes were transfected with siRNA oligo targeting NOD1 or non-targeting control for 24 hr, followed by treatment with or without C12-iEDAP or ISO for 24 hr. NOD1 mRNA expression was evaluated to indicate the knockdown efficiency (A) and glycerol release was measured in the assay buffer. Data are mean±SE (n=3). *, **, indicate significant changes compared to siNT control with $p<0.05$ and $p<0.01$, respectively.

The role of the lipolytic pathway components on NOD1-mediated lipolysis

PKA/HSL lipolytic pathway has been implicated in LPS-induced lipolysis in human primary adipocytes (21). Next, we examined whether NOD1-mediated lipolysis is through the PKA/HSL lipolytic pathway. With pretreatment of PKA inhibitor H-89 (20 μM) or HSL inhibitor CAY10499 (20 μM) for 1 hr followed by co-treatment with stimulants, both C12-iEDAP and LPS-induced lipolysis were significantly attenuated (Fig. 3) ($p<0.001$), demonstrating that both NOD1- and TLR4-mediated lipolysis involve the PKA/HSL lipolytic pathway.

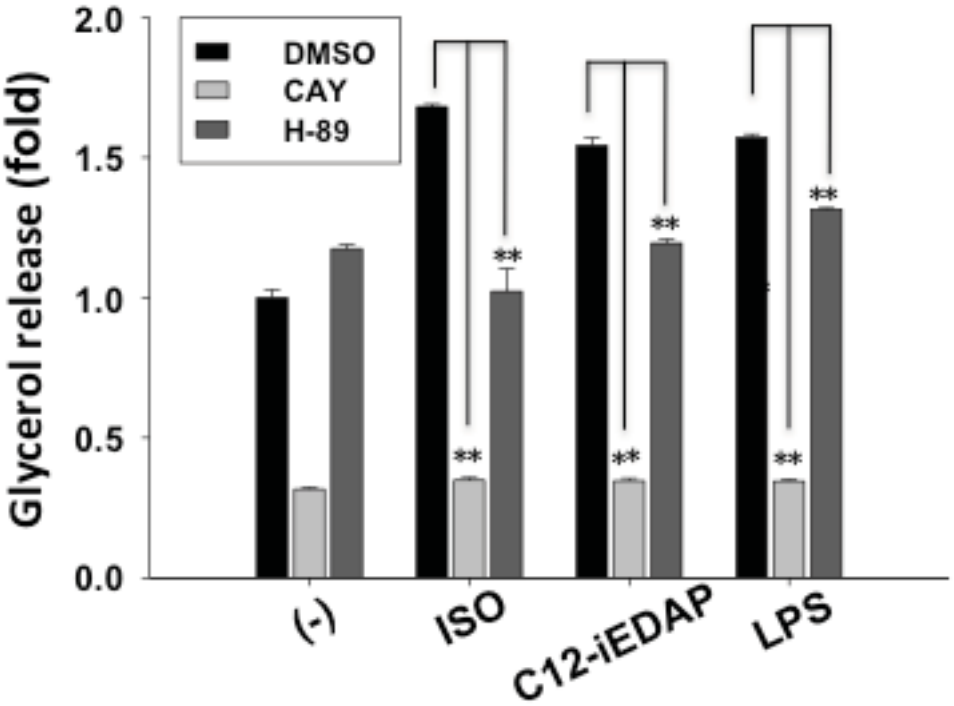

Figure 2.3 Effects of the pharmacological inhibitor of HSL and PKA on NOD1-mediated lipolysis in 3T3-L1 adipocytes. 3T3-L1 adipocytes were pretreated with HSL inhibitor CAY10499 (CAY) (A) or PKA inhibitor H-89 (B) for 1 hr followed by co-treatment with the stimulants for 24 hr. Glycerol release in the cell supernatant was measured. Data are mean±SE (n=3). *, **, indicate significant changes compared to DMSO control with $p<0.05$ and $p<0.01$, respectively.

NOD1 activation suppresses PLIN protein expression

To gain further insight into the molecular mechanisms underlying NOD1-mediated lipolysis, we analyzed the protein expression of the key components of the lipolytic pathways upon NOD1 and TLR4 activation for 8 hrs and 24 hrs. TLR4 activation by LPS modestly increased the protein levels of ATGL and HSL after 24 hr treatment, consistent with a previous report (20). In contrast, NOD1 activation by C12-iEDAP did not increase protein expressions of the ATGL and MGL. Even though C12-iEDAP modestly enhanced HSL protein expression at 8hr, but suppressed it at 24hr (Fig. 4). MGL protein levels were unchanged by LPS. NOD1 activation significantly suppressed PLIN protein by ~ 40% at 24 hr. TLR4 activation by LPS also suppressed PLIN protein expression to a similar degree, consistent with the previous report (20) (Fig. 4).

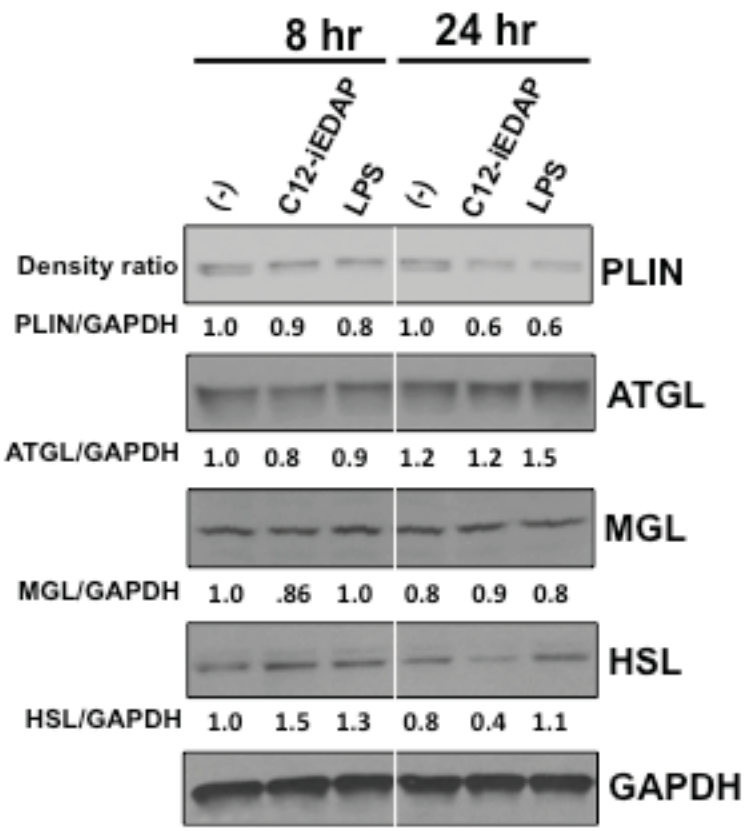

Figure 2.4 NOD1 activation by C12-iEDAP suppresses PLIN protein expression. 3T3-L1 adipocytes were treated with C12-iEDAP, LPS, or the vehicle control for 8 and 24 hr and the whole cell lysate was prepared and analyzed for indicated protein expression by western blot analysis. The intensity of bands were quantified by densitometry and expressed as ratio of indicated protein to the loading control. The ratio was set to be to 1 for the control.

The roles of NF-κB and MAPK pathways on NOD1-mediated lipolysis in 3T3-L1 adipocytes

We have shown that NOD1 stimulation induces activation of both NF-κB and MAPK pathways, leading to proinflammatory chemokines and cytokines expression in 3T3-L1 adipocytes (22). We next examined the involvement of these pathways on NOD1-mediated lipolysis using pharmacological inhibitors of NF-κB and MAPK pathway. With pretreatment of NF-κB inhibitor caffeic acid phenethyl ester (CAPE, 10 μM) and QNZ (10 μM) for 1hr followed by co-treatment with the stimulants, we show that CAPE and QNZ significantly inhibited the lipolysis induced by C12-iEDAP and LPS ($p<0.001$) (Fig. 5A). Interestingly, QNZ, but not CAPE, also inhibited ISO-induced lipolysis. In contrast, pretreatment of JNK inhibitor SP600125 (10 μM), p38 inhibitor SB203580 (2 μM), or ERK inhibitor PD98059 (10 μM), followed by co-treatment with the stimulants, did not significantly affect lipolysis induced by C12-iEDAP (Fig. 5B). Similar results were observed for TLR4 activation by LPS, except that only the inhibitor of ERK modestly suppressed LPS-induced lipolysis. These results demonstrate that the involvement of the NF-κB pathway, but not the MAPK pathway, in NOD1-mediated lipolysis.

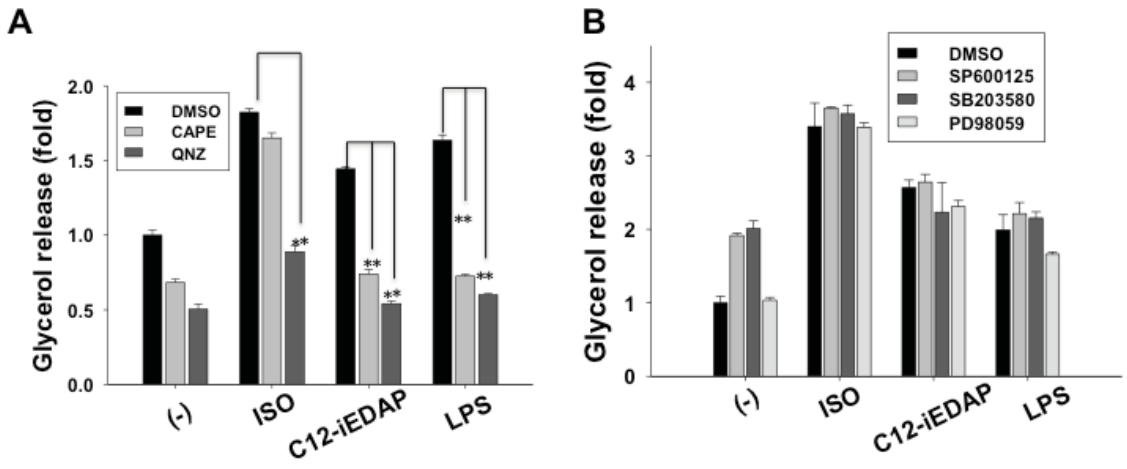

Figure 2.5 Effects of the pharmacological inhibitors of NF-κB and MAPK pathways on NOD1-mediated lipolysis in 3T3-L1 adipocytes. 3T3-L1 adipocytes were pretreated with NF-κB inhibitor caffeic acid phenethyl ester (CAPE), QNZ (A) or the ERK inhibitor PD98059, p38 inhibitor SB203580, and JNK inhibitor SP600125 (B) for 1 hr followed by co-treatment with the stimulants for 24 hr. Glycerol release in the cell supernatant was measured. Data are mean±SE (n=3). *, **, indicate significant changes compared to DMSO control with $p<0.05$ and $p<0.01$, respectively.

Involvement of NF-κB and MAPK pathways in the suppression of PLIN protein by NOD1 activation

Since activation of TLR4 and NOD1 both significantly suppressed PLIN protein expression at 24 hr, we wanted to test whether suppression of PLIN protein expression was mediated by NF-κB or MAPK pathway downstream of the PRR activation. Therefore, we examined the effects of PLIN protein expression by C12-iEDAP or LPS treatment in the presence or absence of the pharmacological inhibitors of NF-κB or MAPKs. Although NF-κB inhibitor CAPE and QNZ attenuated NOD1-mediated lipolysis, they did not significantly reverse the suppression of PLIN protein expression (Fig.6). In fact, CAPE or QNZ alone suppressed the PLIN protein level (Fig. 6). Similarly, CAPE and QNZ also did not reverse LPS-induced suppression of PLIN protein level (Fig.6).

Although all MAPK inhibitors had no or minimal impact on NOD1-mediated lipolysis, the inhibitor of p38 MAPK, but not the inhibitor of ERK or JNK, reversed the suppression of PLIN protein expression by C12-iEDAP (Fig. 7). In contrast, all these inhibitors did not have an effect on LPS-induced suppression of PLIN protein in 3T3-L1 adipocytes (Fig. 7).

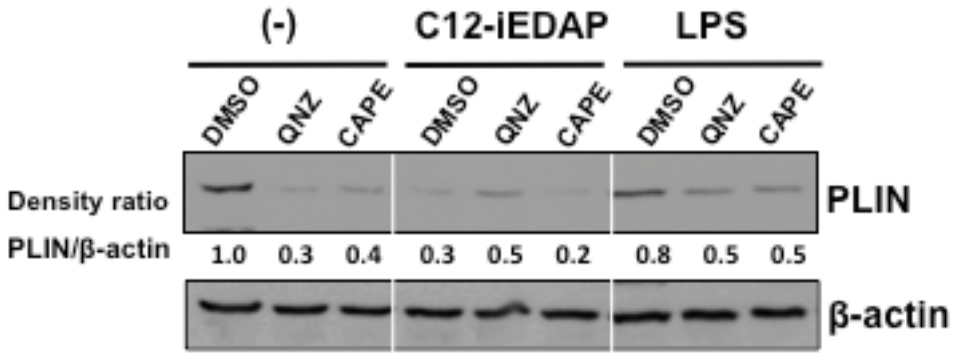

Figure 2.6 The involvement of NF-κB on C12-iEDAP-induced PLIN suppression. 3T3-L1 adipocytes were pretreated with NF-κB inhibitors CAPE, QNZ or the vehicle control for 1hr and then co-treated with the stimulants for 24 hr. The whole cell lysate was prepared and analyzed for PLIN protein expression by western analysis. The intensity of bands were quantified by densitometry and expressed as ratio of indicated protein to the loading control. The ratio was set to be to 1 for the control.

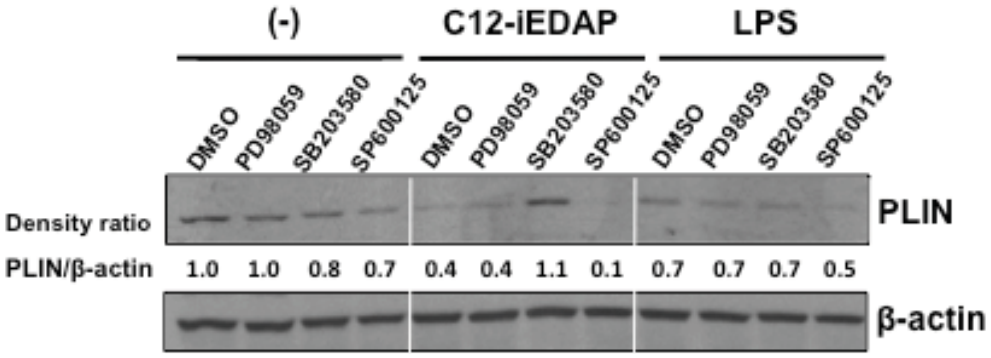

Figure 2.7 The involvement of MAPK on C12-iEDAP-induced PLIN suppression. 3T3-L1 adipocytes were pretreated with ERK inhibitor PD98059, p38 inhibitor SB203580, and JNK inhibitor SP600125 for 1 hr and then co-treated for the stimulants for 24 hr. The whole cell lysate was prepared and analyzed for PLIN protein expression by western analysis. The intensity of bands were quantified by densitometry and expressed as ratio of indicated protein to the loading control. The ratio was set to be to 1 for the control.

DISCUSSION

Accumulating evidence supports that obesity is associated with chronic inflammation. Two families of PRRs, TLR and NLR family, play critical roles in mediating inflammation in adipocytes. Activation of TLR4 or NOD1, have shown to induce inflammatory cytokine/chemokine expression and insulin resistance (17, 25). Moreover, TLR4 activation also directly promotes lipolysis in adipose tissue and adipocytes (20). Here we show that activation of NOD1 induced lipolysis in mature 3T3-L1 adipocytes. Co-treatment with the PKA inhibitor H-89, or the potent inhibitor of HSL CAY10499, abolished the lipolytic effects of NOD1 activation (Fig. 3) and co-treatment with inhibitors of NF-κB also inhibited NOD1-mediated lipolysis (Fig. 5), suggesting that both NF-κB and the lipolytic PKA/HSL pathways are involved in NOD1-mediated lipolysis.

Similar to LPS/TLR4 activation, the robust induction of lipolysis mediated by NOD1 occurs only after 24hr of stimulation (Fig. 1) whereas the adrenergic agonist isoproterenol induces marked lipolysis as early as 6 hr. It has been suggested that in the basal state, ATGL, perilipin, and the ATGL co-activator CGI-58/ABHD5 form a complex at the surface of the LD. The interactions among these proteins limit basal ATGL-mediated lipolysis. HSL is inactive in the cytosol in basal state. In the stimulated state, HSL is phosphorylated by protein kinases (e.g., PKA); the active form of HSL then migrates to the LD surface. PK also activates PLIN that undergoes structural modification and rearrangement leading to fragmentation of the LD. Furthermore, CGI-

58/ABHD5 released from activated PLIN activates ATGL to initiate TAG hydrolysis (26, 27). Our results that NOD1-mediated lipolysis involves PKA/HSL as well as the NF-κB pathway are consistent with the report by Grisouard *et al* on LPS/TLR4-mediated lipolysis in human primary adipocytes (21). These findings raise the question of how the two pathways are linked together to induce enhanced lipolysis. Both TLR4 activation (21) and NOD1 activation (22) induce upregulation of proinflammatory cytokines (e.g., TNF-α), which are known to induce lipolysis (28), from the adipocytes. It is possible that PRR-mediated lipolysis is, directly or indirectly, induced via cytokines, adipokines or other pathways. The fact that NF-κB inhibitors attenuated the lipolysis induced by both NOD1 and TLR4 activation supports the possibility. Further investigations are needed to delineate pathways underlying PRR-mediated lipolysis.

We have reported that both NF-κB and MAPK pathways are activated downstream of NOD1 in 3T3-L1 adipocytes (22). In contrast to the NF-κB pathway, inhibitors of p38, JNK and ERK did not attenuate NOD1-mediated lipolysis and only the ERK inhibitor, PD98059, modestly inhibited LPS-induced lipolysis (Fig. 5). Compared to the significant effects reported for the ERK inhibitor on LPS-induced lipolysis in murine primary adipocytes (20), this may reflect the differences between the cell line we used and the primary adipocytes used in the report.

Perilipin, a lipid droplet coating protein, functions as a barrier to restrict lipase hydrolysis of triglycerides. Perilipin down-regulation could impair the barrier function, thus

facilitating lipolysis. It has been shown that LPS down-regulated perilipin protein in adipose tissues and murine primary adipocytes (20). Similarly, we show NOD1 activation also induced down regulation of perilipin protein (Fig. 4). However, downregulation of perilipin by NOD1 activation did not seem to involve NF-κB pathway, as the two NF-κB inhibitors QNZ and CAPE did not reverse the suppression of perilipin. In fact, each inhibitor alone suppressed perilipin protein (Fig. 6). In contrast, inhibition of p38 MAPK, but not JNK or ERK, reversed the suppression of perilipin by NOD1 activation, suggesting that p38 MAPK is involved in the downregulation of perilipin protein by NOD1 activation by C12-iEDAP (Fig 7). Both the NF-κB and MAPK inhibitors did not reverse the suppression of PLIN protein by TLR4 activation (Fig.6 and 7). Together, these results suggest that the downregulation of PLIN may be common to PRR activation; however, the underlying mechanisms seem to be different and the role of downregulation of perilipin in PRR-mediated lipolysis needs to be investigated further.

Taken together, we show NOD1 activation induces lipolysis in mature 3T3-L1 adipocytes via both PKA/HSL and NF-κB pathways. In addition, NOD1 activation induces down regulation of lipid binding protein Perilipin, similarly to that of TLR4 activation; however, the mechanisms underlying the suppression of PLIN are different between NOD1 and TLR4. Overall, our results demonstrate a novel role of NOD1 activation in adipocytes, suggesting NOD1 activation may contribute to dyslipidemia via enhanced lipolysis.

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VITA

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